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EFFECTS OF DIETARY BIOTIN ON LIVER PYRUVATE
CARBOXYLASE AND METABOLISM OF POULTRY

by



AVTAR SINGH ATWAL

A THESIS

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DEPARTMENT OF ANIMAL SCIENCE

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The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies for acceptance, a thesis entitled
"Effects of Dietary Biotin on Liver Pyruvate Carboxylase and Metabolism
of Poultry" submitted by Avtar Singh Atwal, B.Sc.(Agr.), M.Sc.(Agr.),
in partial fulfilment of the requirements for the degree of Doctor of
Philosophy.

ABSTRACT

Experiments were conducted to develop a biochemical method for assessing the status of biotin in the nutrition and metabolism of poultry and, by use of the method, to study the problem of biotin deficiency in poultry. On the assumption that pyruvate carboxylase (PC) would be the most critical of the biotin-dependent enzymes because it supplies a variety of metabolic products and, therefore, should respond to biotin supply, it was decided to use the measurement of activity of this enzyme in livers as a means of studying the influence of dietary biotin on the metabolism of poultry.

At the outset an assay for PC activity based on the procedure of Utter and Keech (1963) was developed and standardized for use throughout this study.

When adequate biotin was fed to chicks, PC activity per gram of liver increased considerably during the second week of age and remained relatively high until four weeks of age. When a ration deficient in biotin was fed liver PC activity gradually decreased to very low levels at four weeks of age. Chick liver PC activity responded linearly to increasing biotin levels in the ration between 80 and 320 $\mu\text{g}/\text{kg}$. The pattern of appearance of deficiency symptoms and the relation of biotin supply to PC activity in livers suggested that usage of biotin in chicks for alternate functions may have priorities depending upon the extent of its supply.

The performance of turkey poult's and PC activity in their livers were affected by the protein supplement used, biotin level of the ration and progression of the hatching season. When meat meal and fish meal were the only protein supplements used, symptoms of a biotin deficiency occurred, rate of growth was reduced and PC activity of liver

was low. The addition of biotin to the ration largely alleviated the symptoms of the deficiency and increased PC activity in livers but failed to improve rate of growth of poult. When soybean meal was included in the protein supplement, rate of growth was high, no symptoms of a biotin deficiency were evident and PC activity was increased considerably. Liver PC activity was higher in early hatched than in late hatched poult, when rations containing soybean meal were fed.

Liver PC activity of chicks and poult responded to levels of biotin higher than those capable of eliciting a growth response. It was suggested that use of higher levels of biotin than those considered necessary for optimal growth might be desirable.

A deficiency of biotin resulted in a marked decrease in the PC activity and in the in vivo incorporation of ^{32}P into RNA and DNA in livers of chicks, however, when suboptimal levels of biotin (80 or 120 $\mu\text{g}/\text{kg}$) were available, no relationship between PC activity and ^{32}P incorporation into nucleic acids of chick liver was noted.

It was hypothesized that a dietary supply of tricarboxylic acid cycle intermediates might serve as a source of oxaloacetate, thus reducing the dependence of chicks on their liver PC and thereby serving as a tool to investigate the possible metabolic lesion resulting from biotin inadequacy. The inclusion of 4% sodium succinate and 6% citric acid into a well balanced ration proved deleterious for growing chicks but 4% sodium succinate alone was well tolerated. The inclusion of 2 or 4% sodium succinate in rations containing 80 and 120 μg of biotin/kg did not have any consistent effect on PC activity or ^{32}P incorporation in chick liver.

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INTRODUCTION

During the past twenty years, turkey growers in Alberta have experienced sporadic outbreaks of a syndrome in poult's of 2-4 weeks of age. The condition has been characterized by dermatitis, broken feathers, perosis, diarrhoea, slow growth rate and increased mortality. The severity of the disorder seemed to increase as the hatching season progressed. Recently, outbreaks of a similar syndrome have also been reported in the U.S.A.

Early experiments conducted at the University of Alberta showed that the disorder could be alleviated to a considerable extent by adding biotin to the rations fed but, in some instances, biotin therapy was not fully effective. Moreover, calculation of the biotin content of rations, using published values for various ingredients, indicated that there should have been enough biotin to meet the requirement of poult's. It has therefore been difficult to ascertain whether lack of biotin was the primary cause of the syndrome or whether other factors were involved. Consequently it was considered that more extensive studies should be undertaken to establish the role that biotin plays in the occurrence of the syndrome.

It was anticipated that an insufficiency of biotin may result in metabolic lesions at the level of biotin dependent enzymes and that pyruvate carboxylase (PC) would be the most critical of these because it supplies oxaloacetate for replenishing the tricarboxylic acid (TCA) cycle intermediates that are withdrawn for the synthesis of a variety of cell constituents and metabolic products. It therefore seemed possible that a deficiency of biotin in the ration would have a marked effect on levels of PC in the tissues of animals. The experiments

reported here were undertaken to develop a suitable assay procedure for estimating PC activity in crude preparations of liver and, by means of the assay procedure, to assess the relationships between biotin levels of the ration and PC activity in livers of chicks and pourets. Also the effect of adding some TCA cycle intermediates to the ration on the status of biotin in the metabolism of chicks was investigated.

REVIEW OF LITERATURE

A. History

The discovery that biotin is an essential vitamin resulted from studies designed to prevent or cure deficiency symptoms in experimental animals and from growth studies with microorganisms. Investigations of the nature of the toxicity of raw egg white in higher animals led to the demonstration of a protective factor (factor X) in potatoes, liver and yeast (Boas, 1927). This factor was found in several other foodstuffs and was named vitamin H by György (1931—cited by Ochoa and Kaziro, 1965) and 'anti-egg-white injury factor' by Lease and Parsons (1934). Allison, Hoover and Burk (1933) and Allison and Hoover (1934) reported that a factor isolated from yeast was required for respiration and growth of several strains of 'Rhizobia'. This factor was called 'Coenzyme R'. Kogl and Tönnis (1936) isolated a fraction from crystalline egg yolk, which was an extremely potent stimulant for the growth of yeast. They called this active substance, biotin.

Classical biochemical investigations eventually led to the conclusion that biotin was identical with 'Coenzyme R' (West and Wilson, 1939); with vitamin H (György et al., 1940) and with the antidermatitis factor for poultry (Hegsted et al., 1940, 1942; Ansbacher and Landy, 1941). Since then, biotin has been reported to be required for growth of a wide variety of yeasts, fungi, bacteria, algae, protozoa, insects, birds and mammals as reviewed by Briggs (1961) and Langer and György (1968).

B. Biotin in Poultry Nutrition

Ringrose, Norris and Heuser (1931) first reported pellagra-like symptoms in chicks fed purified rations containing dried egg albumin or purified casein. The symptoms described included lesions

around the eyes, encrustations at the beak angle and severe lesions on the feet. The outer layers of the skin on the bottom of the feet and between the toes peeled off and cracks and fissures developed. The remaining skin layers thickened and cornified.

Lease and Parsons (1934) and Hegsted et al. (1940) failed to prevent or cure the new syndrome by administering abundant amounts of pantothenic acid concentrates and pantothenic acid, respectively. However, feeding potent sources of biotin such as extracts of yeast, liver and kidney (Hegsted et al., 1940); molasses (Hegsted et al., 1940; McElroy and Jukes, 1940); dried rumen contents from a cow (McElroy and Jukes, 1940) as well as concentrates and crystalline biotin (Ans�acher and Landy, 1941) prevented the new syndrome completely and improved growth rate of chicks.

The results of a number of studies demonstrated that symptoms of a biotin deficiency were distinct from those of a pantothenic acid deficiency. Hegsted et al. (1940), McElroy and Jukes (1940) and Ans�acher and Landy (1941) noted that in a biotin deficiency, lesions (usually referred to as dermatitis) appeared on the feet before mandibular lesions were evident whereas, in a pantothenic acid deficiency, lesions were first noted around the eye and at the beak angle.

In addition to dermatitis, appearance of perosis (also known as hock disorder or slipped tendons) has been reported in chicks fed raw egg white (Ringrose et al., 1931; McElroy and Jukes, 1940) and chicks fed biotin-deficient simplified rations (Jukes and Bird, 1942). Jukes and Bird (1942) reported that perosis was cured completely by injecting very small amounts of biotin but dermatitis was only partially relieved.

Turkey poulets appear to be much more susceptible to a deficiency of biotin than chicks. Patrick et al. (1941) and Patrick, Boucher and Knadel (1942) reported the occurrence of dermatitis and perosis in growing poulets fed simplified diets or even commercial-type rations. Deficiency symptoms were prevented or cured by supplementing the rations with liver residues, yeast or biotin.

Sporadic outbreaks of a disorder characterized by poor growth, broken feathers, dermatitis, perosis, and high mortality have been reported in turkey flocks, raised on practical rations (McGinnis and Carver, 1947; Robblee and Clandinin, 1953; Slinger and Pepper, 1954; Johnson, 1967; Misner, 1967; Waibel, 1968). Robblee and Clandinin (1953) reported that the addition of calcium pantothenate and biotin to practical starter rations prevented the disorder.

Symptoms of a biotin deficiency usually appear at two to four weeks of age (Patrick et al., 1942; Robblee and Clandinin, 1953; Dobson, 1967; Misner, 1967) but under marginal biotin supply may be cured after the fourth week. The probability exists, however, that a deficiency of biotin in the early growth period may result in leg weakness at a later stage of growth (Johnson, 1967; Jensen and Martinson, 1969).

Many workers have investigated the biotin requirements of poultry. Ansbacher and Landy (1941) and Hegsted et al. (1942) suggested that the minimal curative dose for chicks was approximately 100 $\mu\text{g}/\text{kg}$ of ration. Patrick et al. (1942) suggested that the biotin requirement of chicks and poulets was 2 and 5 $\mu\text{g}/\text{day/bird}$, respectively, for the first four weeks. However, Slinger and Pepper (1954) found that a ration containing 280 μg biotin/kg, which supplied 8.4 μg biotin/poult/day, was insufficient in the absence of antibiotics. Waibel et al. (1967)

have suggested biotin requirements of 208 μg and 187 μg per kg for prestarter and starter rations for turkeys, respectively. Jensen and Martinson (1969) have reported that 284 μg of biotin/kg of ration gave optimal growth of poult's in one experiment but in the second experiment a higher level of supplementation appeared to be necessary.

Reasons advanced for observed variations in the biotin requirements of poultry include availability of biotin in the feed and the composition of the ration fed. Patrick et al. (1942) suggested that all of the biotin of feedstuffs may not be available to birds; Slinger and Pepper (1954) remarked that availability of biotin from different feedstuffs may vary and Wagstaff, Dobson and Anderson (1961) found the biotin content of barley and hulless barley to be 130 μg and 100 $\mu\text{g}/\text{kg}$, respectively, by microbial assays, whereas chick assay gave the value of 35 $\mu\text{g}/\text{kg}$ in both cases. In addition it has been reported that the nature of the carbohydrates in the ration may be an important factor in determining biotin requirement of poult's (Couch et al. 1948, 1949; Dobson, 1967). Thus it may be concluded that microbial assays for biotin may fail to represent the net effect of the ration.

C. Metabolic Roles of Biotin

(i) Implication of Biotin in Carboxylation

Soon after the nutritional significance of biotin was recognized, studies were undertaken to establish the biochemical role of biotin. Early investigations indicated that biotin might be involved in carbohydrate metabolism, especially in pyruvate utilization. Summerson, Lee and Partridge (1941) and Pilgrim, Axelrod and Elvehjem (1942) showed that utilization of pyruvic acid by liver slices or liver homogenates from biotin-deficient rats was much lower than in the

case of normal rats. Growth replacement studies showed that aspartic acid could partially substitute for the growth stimulating effect of biotin for various microorganisms (Köser, Wright and Dorfmann, 1942; Stokes, Larsen and Gunness; 1947, 1947a). Potter and Elvehjem (1948) showed that aspartic acid and oleic acid could almost completely replace biotin for the growth of L. arabinosus and that the biotin requirement for aspartic acid synthesis was at least 10 times greater than that for other functions. The studies mentioned above suggested that biotin was probably required for some reaction involved in the metabolic transformation of pyruvate to aspartate. Stokes et al. (1947a) presented evidence that biotin was not required for the transamination of aspartate and somewhat presumptive evidence that it might be involved in the Wood-Werkman reaction.

Within a few months reports from four laboratories, employing different techniques and organisms, independently suggested that biotin was involved in the decarboxylation of oxaloacetic acid and that under some conditions this process was reversible. Lardy, Potter and Elvehjem (1947) conducted growth replacement studies on L. arabinosus 17-5; Shive and Rogers (1947) employed an inhibition analysis technique with E. coli. and L. arabinosus; Lichstein and Umbreit (1947) worked with E. coli and employed a variety of enzymatic techniques and Ochoa et al. (1947) used biotin-deficient turkeys and enzymatic techniques. Thus it was inferred that biotin was involved in carbon dioxide fixation.

Further evidence that biotin might be involved in carbon dioxide fixation was presented by Wessman and Werkman (1950) who showed that cell free extracts of M. lysodekticus from biotin deficient cultures gave far less ^{13}C exchange between bicarbonate- ^{13}C and

oxaloacetate than the extracts from normal cultures. Addition of avidin (an inhibitor of biotin) to cell free extracts from normal cultures prevented fixation of $^{13}\text{CO}_2$ and production of oxaloacetate.

Several attempts to correlate the bound biotin of oxaloacetic decarboxylase preparations with their activity were unsuccessful (Vennesland, Gollub and Speck, 1949; Byerrum, Brown and Ball, 1950; Herbert, 1950; Plaut and Lardy, 1950). This paradox led to the hypothesis that biotin might be involved in the synthesis of carboxylases and decarboxylases rather than functioning as a prosthetic group (Blanchard *et al.* 1950). However, Lichstein (1955, 1957) succeeded in establishing a highly significant correlation between the degree of purity of oxaloacetic carboxylase extracted from chick liver and the quantity of biotin which could be released by acid hydrolysis. The purest enzyme preparation reported contained 2.44 μg of biotin/g of protein (Lichstein, 1957). Although this proportion of biotin to enzyme protein was very low, it clearly indicated that biotin might function as a coenzyme for oxaloacetic carboxylase.

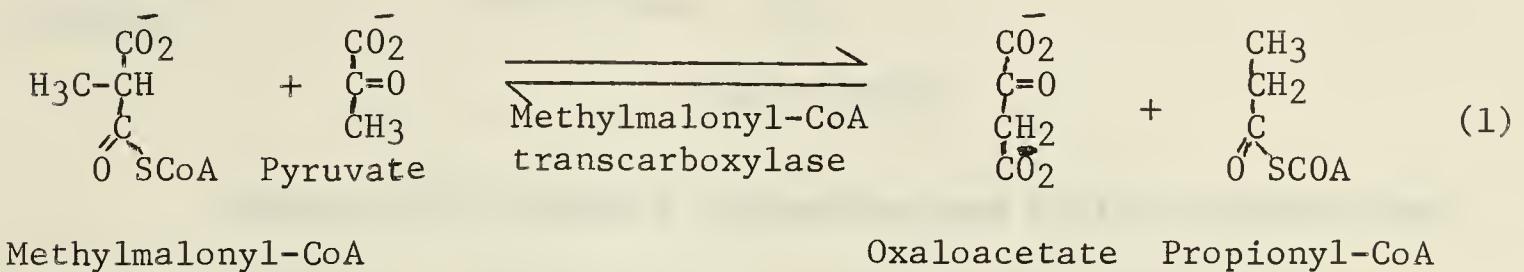
(ii) Biotin Containing Enzymes

During the 1950's most studies on the biochemistry of biotin were directed towards establishing its role as a coenzyme. Five enzymes have been conclusively shown to contain biotin and there is some evidence suggesting that a sixth enzyme may also be biotin dependent. A brief review of their discovery, relationship with biotin and metabolic functions is presented in the following sections:-

(a) Methylmalonyl-CoA Transcarboxylase (E.C.2.1.3.1)

The biotin dependence of propionic acid bacteria for proper decarboxylation of succinate to propionate was established by

Delwiche (1950) and Lichstein (1950). Swick and Wood (1960) showed that avidin inhibited this transformation and that methylmalonyl-CoA was an intermediate in this process. Stjernholm and Wood (1961) isolated the enzyme methylmalonyl-CoA transcarboxylase which catalyzed the reaction shown in Equation 1.



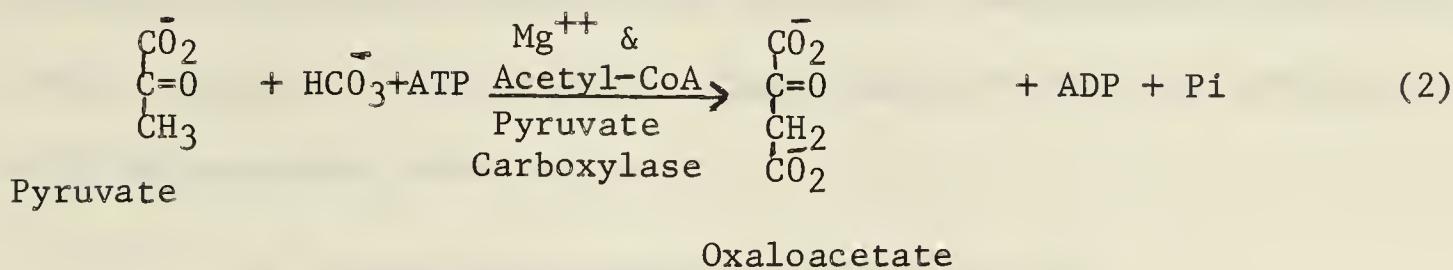
Wood *et al.* (1963) labelled the enzyme with tritiated biotin by growing propionic acid bacteria in a medium containing tritiated biotin and found that homogeneous enzyme preparations contained 1.5 mg biotin/g of protein.

In propionic acid bacteria this enzyme catalyzes an important reaction involved in the utilization of pyruvate but no animal or avian tissue has been reported to contain any appreciable amount of methylmalonyl-CoA transcarboxylase.

(b) Pyruvate Carboxylase (E.C.6.4.1.1)

Although the carboxylation of pyruvate *per se* to form oxaloacetate had been postulated many times, until 1950, there was little direct experimental evidence to support this hypothesis. Kaltenbach and Kalnitsky (1951) reported that crude extracts of E. coli and P. morganii formed small amounts of oxaloacetate from pyruvate. Woronick and Johnson (1960) showed that, in the presence of pyruvate, ATP and CO₂, cell free extracts of A. niger formed aspartate, malate and fumarate, suggesting the existence of ATP-dependent carboxylation. The same year

Utter and Keech (1960) presented conclusive evidence for the existence of a pyruvate carboxylase system in the mitochondria of avian and beef liver which catalyzed the reaction shown in Equation 2.



Evidence that pyruvate carboxylase was biotin dependent was presented by Keech and Utter (1963), who showed that the enzyme was inhibited by avidin. Scrutton and Utter (1965) showed that pyruvate carboxylase contained biotin and the maximal biotin content of the purified preparation was 1.45 mg/g of protein.

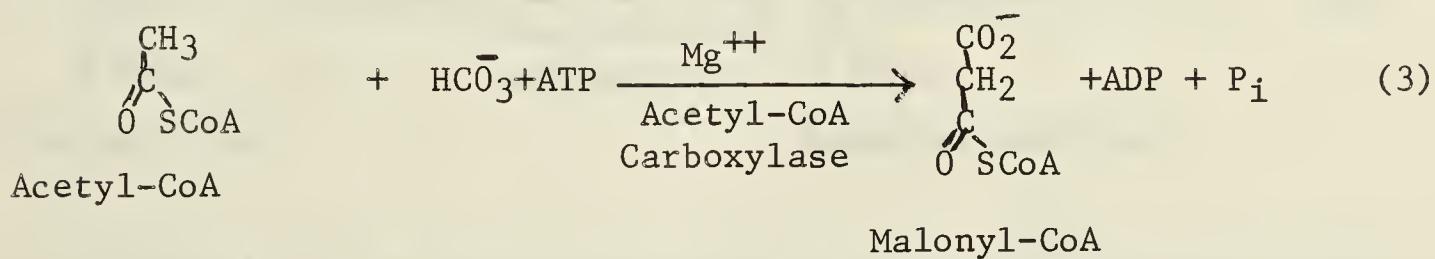
Pyruvate carboxylase has been found to occur in liver and kidney tissue of various mammalian and avian species (Keech and Utter, 1963; Ling and Keech, 1966); in adipose tissue of mammals (Ballard and Hanson, 1967); in the mammary gland of mammals (Gul and Dils, 1969); in brain tissue of mammals (Salganicoff and Keoppe, 1968) and in various microorganisms (Seubert and Remberger, 1961; Bloom and Johnson, 1962; Benedict, 1964; Young and Utter, 1968).

The most important role of pyruvate carboxylase is to supply four carbon (C_4)-dicarboxy acids for replacing the TCA cycle intermediates withdrawn for the synthesis of metabolic products i.e. citrate for fatty acid synthesis; α -ketoglutarate for the synthesis of glutamate and other amino acids; succinyl-CoA for porphyrin synthesis and oxaloacetate for the synthesis of aspartate, pyrimidines and phosphoenolpyruvate (PEP) which is a precursor of hexoses, hexose phosphates, pentose phosphates, and tryptophan (Lowenstein, 1967). It has been

suggested that pyruvate carboxylase is also involved in maintaining reduced pyridine nucleotides in the cytoplasm and thus indirectly may affect lipogenesis (Ballard and Hanson, 1967), gluconeogenesis (Deodhar and Mistry 1969) and ascorbic acid synthesis (Dakshinamurti and Mistry, 1962). Details of the metabolic significance of pyruvate carboxylase will be discussed under Section D.

(c) Acetyl-CoA Carboxylase (E.C.6.4.1.2)

Acetyl-CoA carboxylase was the first enzyme to be recognized as a biotin-containing enzyme. Gibson, Titchener and Wakil (1958) observed that bicarbonate was an absolute requirement for palmitate synthesis from acetyl-CoA and Wakil (1958) showed that malonyl-CoA was an intermediate in fatty acid synthesis. Wakil, Titchener and Gibson (1958) and Wakil and Gibson (1960) showed that one of the enzyme fractions synthesizing fatty acids from acetyl-CoA contained biotin and that avidin inhibited the synthesis of palmitate. They concluded that the enzyme fraction containing biotin was concerned with carboxylation of acetyl-CoA to malonyl-CoA, as presented in Equation 3.



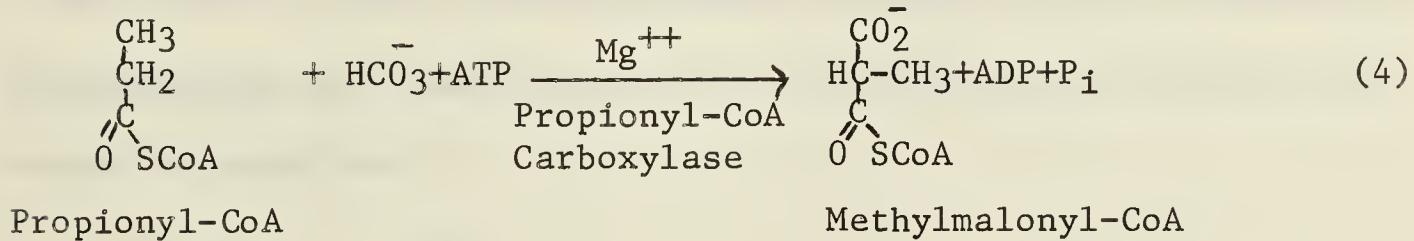
Later acetyl-CoA carboxylase was highly purified from chick liver and found to contain 0.7 mg of biotin/g of protein (Waite and Wakil, 1962).

The effects of biotin deficiency on acetyl-CoA carboxylase activity and lipogenesis have been studied by many investigators. Livers of biotin-deficient rats and chicks had lower levels of acetyl-CoA carboxylase than the controls fed biotin (Wakil and Gibson, 1960). The

incorporation of labelled acetate into body fatty acids was reduced in biotin deficient rats (Gram and Okey, 1958), chicks (Donaldson, 1964, 1967) and pullets (Dalnave and Brown, 1967); but the incorporation of labelled malonate into fatty acids was not affected in biotin deficient chicks (Donaldson, 1964, 1967). Thus it was established that biotin deficiency affected lipogenesis by decreasing the acetyl-CoA carboxylase activity.

(d) Propionyl-CoA Carboxylase (E.C.6.4.1.3)

It has been known for some time that in animal tissues, propionic acid is metabolized via symmetrical C₄-dicarboxy acids (Lardy and Peanasky, 1953). Flavin, Castro-Mendoza and Ochoa (1957) and Flavin and Ochoa (1957) reported that propionic acid was first activated to propionyl-CoA which is then carboxylated, in the presence of ATP to yield methylmalonyl-CoA. The enzyme catalyzing the reaction shown in Equation 4, was isolated and purified from pig heart by Tietz and Ochoa (1959) and named propionyl-CoA carboxylase.



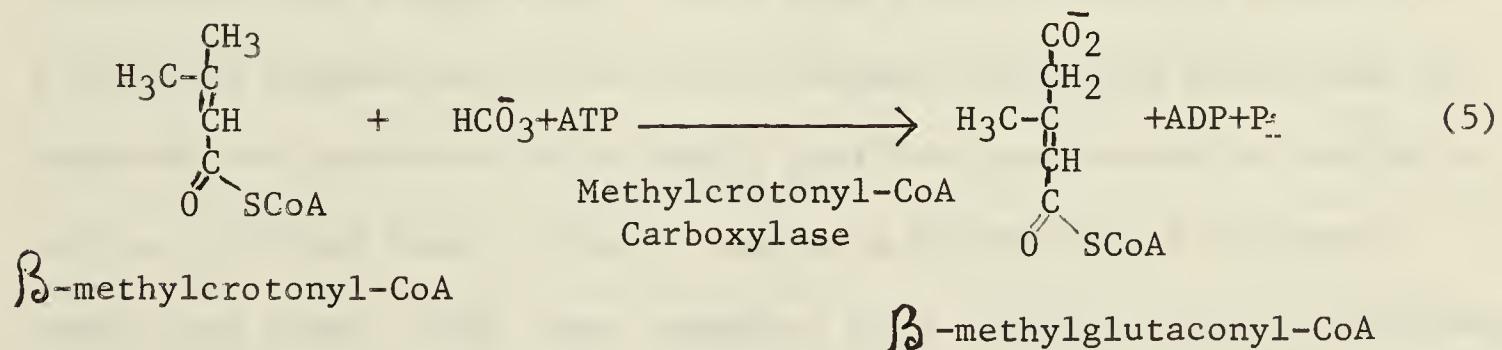
Methylmalonyl-CoA is then transformed to succinyl-CoA and succinate.

Inhibition of propionyl-CoA carboxylase by avidin, demonstrated in pig heart preparations (Kaziro, Leone and Ochoa, 1960) and in bovine liver preparations (Halenz and Lane, 1960) suggested that the enzyme was biotin dependent. Kaziro *et al.* (1961) reported that highly purified propionyl-CoA carboxylase contained 1.34 mg of biotin/g of protein.

The metabolic significance of propionyl-CoA carboxylase was discussed by Flavin and Ochoa (1957) and it was mentioned that in the animal system this enzyme has an important role in the utilization of propionate arising from the degradation of odd number carbon fatty acids and branched chain aliphatic amino acids as well as from the fermentation of carbohydrates in the rumen.

(e) Methylcrotonyl-CoA Carboxylase (E.C.6.4.1.4)

Methylcrotonyl-CoA carboxylase was discovered as a result of investigations of leucine catabolism. Bachhawat, Robinson and Coon (1955, 1956) reported that β -hydroxyisovaleryl-CoA is an intermediate of leucine degradation and that in the presence of partially purified enzymes of mammalian liver and heart, β -hydroxyisovaleryl-CoA is carboxylated in the presence of ATP and CO_2 to yield β -hydroxy- β -methylglutaryl-CoA. Subsequently it was reported that the compound that undergoes carboxylation is β -methylcrotonyl-CoA rather than β -hydroxyisovaleryl-CoA (Knappe, 1957 and Lynen 1958 - cited by Lynen, 1959). These workers purified the enzyme from a species of Mycobacterium and demonstrated that it catalyzes the reaction presented in Equation 5.



Del Campillo-Campbell, Dekker and Coon (1959) and Rilling and Coon (1960) confirmed this finding using enzyme obtained from chick and ox livers.

The dependence of methylcrotonyl-CoA carboxylase on biotin was implicated by Woessner, Bachhawat and Coon (1958), who observed that extracts of biotin-deficient rat liver gave lower carboxylation of β -hydroxyisovaleryl-CoA than those from normal rats fed biotin. Himes *et al.* (1963) purified methylcrotonyl-CoA carboxylase from species of Achromobacter and showed that the purified preparation contained 1.33 mg of biotin/g of protein.

(f) Carbamyl Phosphate Synthetase (E.C.2.7.2.2.)

There has recently been controversy regarding the possibility that carbamyl phosphate synthetase may be a biotin dependent enzyme. Wellner, Santos and Meister (1968) reported that purified carbamyl phosphate synthetase of E. coli was inhibited by avidin and that the enzyme contained 1.46 mg of biotin/g of protein. On the contrary, lack of inhibition by avidin has been reported using frog liver and pigeon liver preparations (Peng and Jones, 1969) or E. coli and beef liver enzymes (Huston and Cohen, 1969). The latter investigators also failed to detect biotin in purified carbamyl phosphate synthetase from frog liver, beef liver, or E. coli by the procedure found adequate for estimating the biotin content of pyruvate carboxylase from these sources. Guthohrlein and Knappe (1968, 1969) also failed to detect biotin in a purified preparation of rat liver carbamyl phosphate synthetase but observed its inhibition by a highly purified preparation of avidin as well as by other basic proteins including protamine and lysozyme. Huston and Cohen (1969) have suggested that carbamyl phosphate synthetase may have some site other than the biotin group, which may bind with basic proteins and render the enzyme inactive. It was suggested that because

of the varying basicity of avidin from preparation to preparation, variations in its binding capacity may be expected. Further investigations are required to resolve the controversy.

(iii) Indirect Effects of Biotin Deficiency

Mammalian and avian species show adaptation to a chronic deficiency of biotin. In severe biotin deficiency many metabolic functions have been reported to be adversely affected. For example biotin deficiency has been reported to impair deamination of aspartate (Lichstein and Umbreit, 1947a), deamination of serine and threonine (Lichstein and Christman, 1948), reductive carboxylation of pyruvate by malic enzyme (Ochoa *et al.*, 1947), tryptophan metabolism (Shanmuga-Sundaram *et al.*, 1954), biosynthesis of citrulline and arginine (MacLeod and Lardy, 1949) and protein biosynthesis (Ahmad, Rose and Grag, 1961). A critical appraisal of the indirect metabolic effects resulting from biotin deficiency and rational explanations for these effects, based on the known functions of biotin have been presented in a review article by Mistry and Dakshinamurti (1964).

D. Metabolic Significance of Pyruvate Carboxylase (PC)

Soon after the discovery of PC and elucidation of the occurrence of both PC and PEP carboxykinase in the mitochondria of chick liver, it was suggested that these two enzymes acting in concert could represent the major pathway for the synthesis of PEP from pyruvate in the process of gluconeogenesis (Keech and Utter 1963). Pyruvate carboxylase has also been recognized as a key gluconeogenic enzyme in animals (Lardy *et al.*, 1964; Lardy, Paekau and Walter, 1965; Shrago and Lardy, 1966; Walter, Paekau and Lardy, 1966). They suggested that

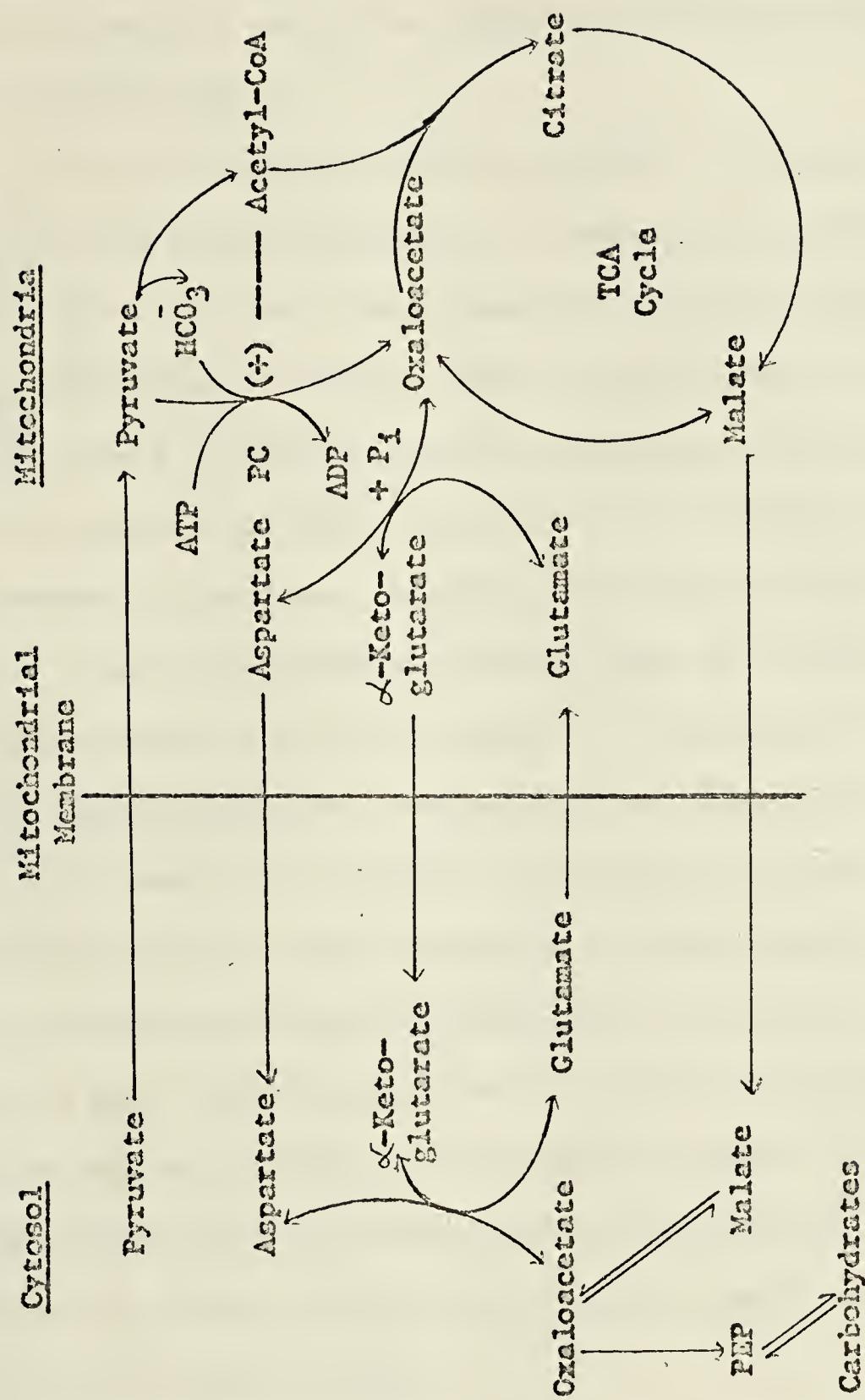


FIG. 1. Synthesis of PEP from pyruvate when PC is in the mitochondria and NADP carboxylase is in the cytosol (adapted from Sirago and Lardy, 1966).

the mechanism shown in Fig. 1, was involved in transporting gluconeogenic precursors across the mitochondrial membrane in tissues such as rat liver, in which PC is located in mitochondria and PEP carboxykinase is present in the cytosol.

Mistry and Deodhar (1968) and Deodhar and Mistry (1969a) have reported that, in biotin-deficient rat liver, gluconeogenesis was blocked at two steps namely those catalyzed by PC and glyceraldehyde-3-phosphate dehydrogenase (G-PDH). They educed evidence to show that these defects were not due to lack of apopyruvate carboxylase or G-PDH activity but rather that the lack of biotin was limiting the formation of holopyruvate carboxylase. Decreased reducing power was limiting the G-PDH step. Lack of PC affected reducing power in two ways; firstly, lack of oxaloacetate limited the operation of the TCA cycle resulting in decreased NADH production, and secondly the NADH pool of the cell was depleted through the conversion of pyruvate to lactate. Decreased reducing power has also been reported to decrease ascorbic acid synthesis in biotin deficiency (Dakshinamurti and Mistry, 1962). Rognstad and Katz (1966) established the operation of pyruvate cycle in the adipose tissue leading to the supply of NADPH for lipogenesis. Ballard and Hanson (1967) have suggested the occurrence of extra-mitochondrial PC in rat adipose tissue and the operation of an extended pyruvate cycle, as shown in Fig. 2.

Earlier observations that biotin was involved in protein biosynthesis have been traced to lack of C₄-dicarboxy acids in biotin deficiency (Dakshinamurti and Mistry, 1963). Buchanan and Hartman (1959) suggested that the effect of biotin deficiency on purine biosynthesis was the result of limited aspartate supply. Mistry and

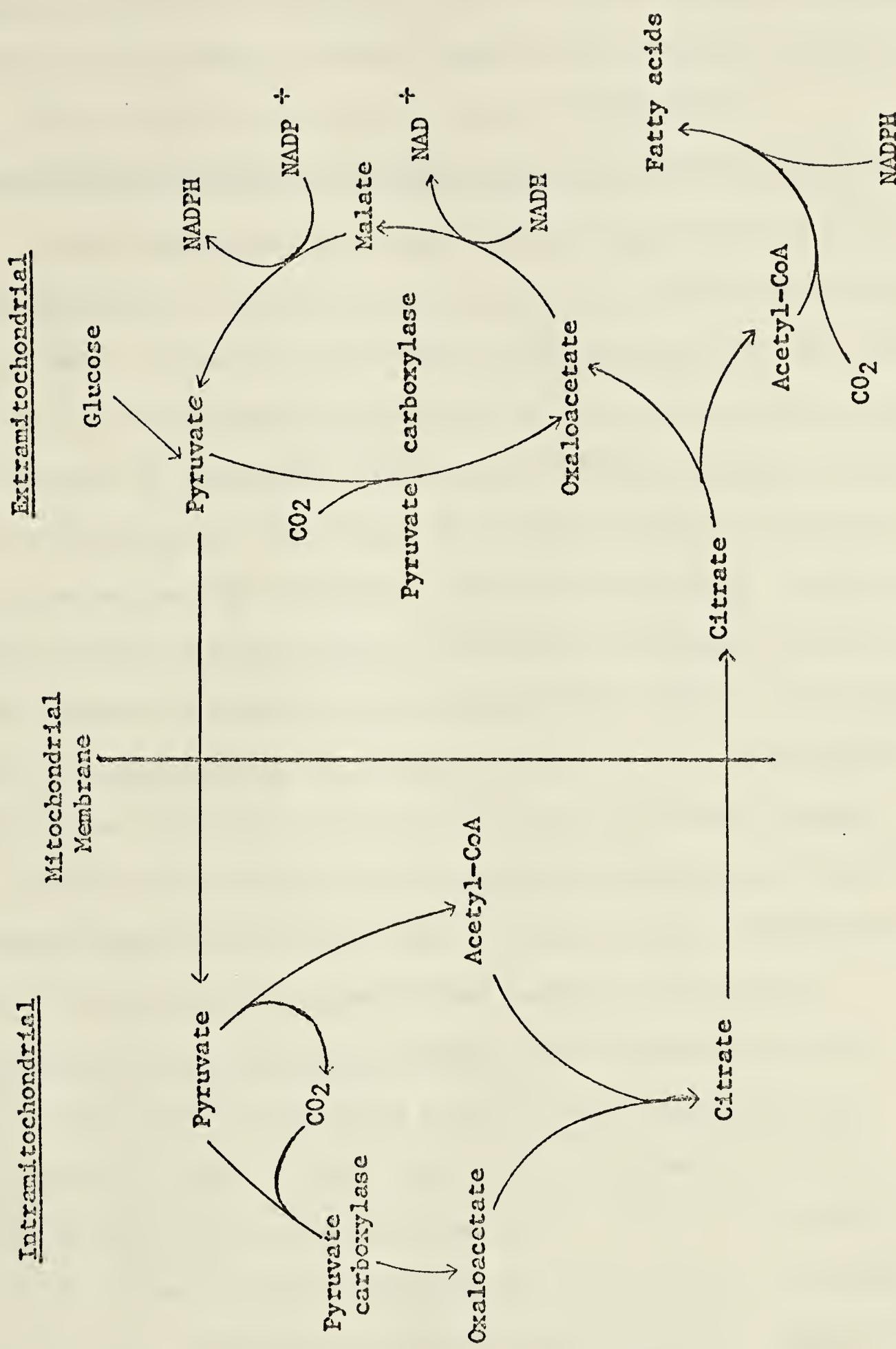


Fig. 2. Pyruvate cycle in rat adipose tissue (adapted from Ballard and Hansson, 1967).

Dakshinamurti (1964) also suggested that many indirect effects of biotin deficiency are the result of chronic adaptation to a reduced aspartate supply. Thus PC is seen to occupy a key role in metabolism.

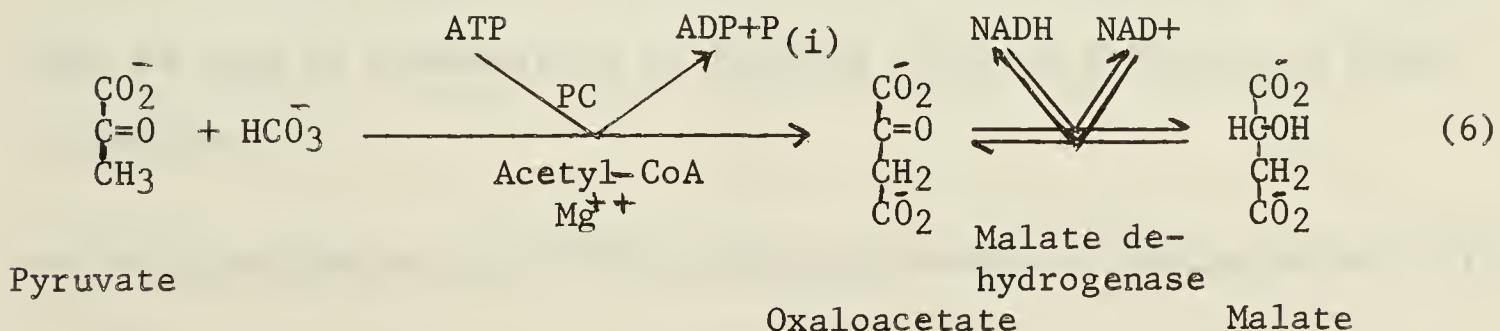
E. Intracellular Distribution and Extraction of Pyruvate Carboxylase (PC)

Some information on the intracellular distribution of PC has been forthcoming from studies involving extraction of PC activity from various tissues. Utter and Keech (1960, 1963) reported that chick liver PC was associated with particulate matter and that mitochondria contained a major portion of the enzyme. Krebs et al. (1963); Freedmann and Kohn (1964) and Lardy et al. (1965) were of the opinion that rat liver PC activity was exclusively localized within the mitochondria. Salganicoff and Koeppe (1968) reported that the PC activity of mammalian brain tissue was also exclusively associated with mitochondria. However, the occurrence of extramitochondrial PC has been suggested in the chick-embryo liver (Rindaudo and Giunta, 1967); in rat liver and kidney (Henning et al., 1966); in rat adipose tissue (Ballard and Hanson, 1967) and in rat mammary gland (Gul and Dils, 1969). Utter and Keech (1960, 1963) extracted PC activity from acetone dried powder or lyophilized preparations of liver and kidney. Wagle (1964) suggested that all the PC activity could be extracted from rat liver mitochondria by homogenizing the tissue at room temperature. He noted that mitochondria obtained from such homogenates did not exhibit any residual PC activity. Other work indicated that PC activity was not completely extracted at room temperature. Freedmann and Kohn (1964) reported that only 40% of the rat liver PC was found in the soluble fraction and another 10% could be measured in intact mitochondria but the remaining 50% was only released by preparing acetone powder of the mitochondria.

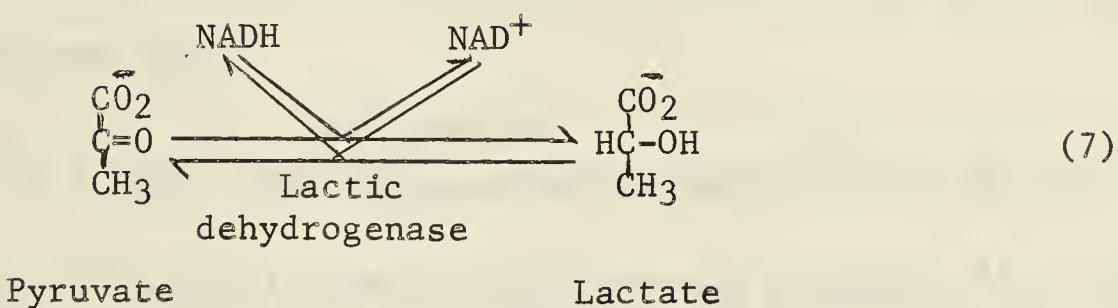
Ballard and Hanson (1967) have also reported that the homogenization of adipose tissue at 25°C did not release all of the PC activity. Gul and Dils (1969) reported that 38% of the PC activity of homogenized mammary gland of the rat was found in particle-free supernatant, whereas the activity of the mammary gland of the rabbit was found exclusively in the particulate material. Salganicoff and Koeppe (1968) suggested the possibility of releasing mitochondrial PC by treating with 0.2% triton X-100 for 1 minute. Böttger *et al.*, (1969) reported that total PC activity from rat liver mitochondria could be solubilized by four successive 10 sec periods of sonication. Using digitonin treatments of isolated rat liver mitochondria, it was shown that PC activity was localized within the matrix space, (Böttger *et al.*, 1969). Thus the available evidence suggests that most of the PC activity is found in the mitochondria although some activity can be readily released from the mitochondria. Whether the release of a portion of PC activity from the mitochondria is an artifact or whether it has some metabolic significance has not been established. It can also be concluded that to release the total PC activity rigorous treatment of tissue is necessary.

F. Estimation of Pyruvate Carboxylase (PC)

For purified preparations of PC, Utter and Keech (1963) described a spectrophotometric assay procedure involving the reactions presented in Equation 6.

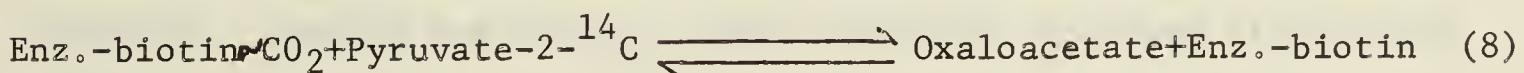


The rate of reaction was calculated by following the utilization of NADH as measured by the decrease in optical density at 340 m μ . The procedure was not suitable for crude liver preparations because of the large amount of lactic dehydrogenase present which catalyzes the reaction shown in Equation 7, thereby causing rapid disappearance of NADH.

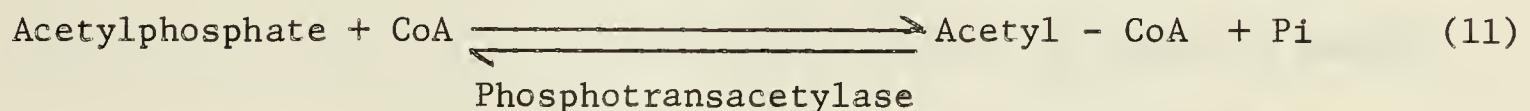
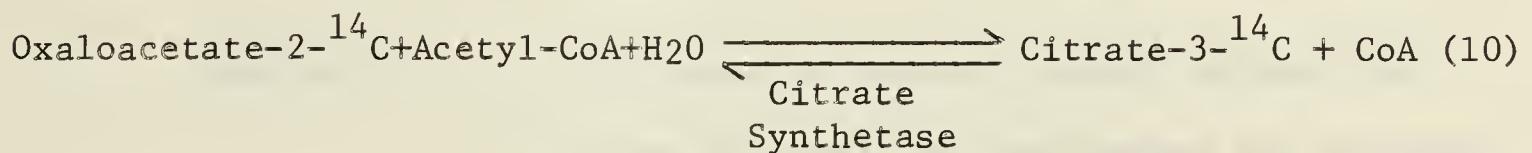
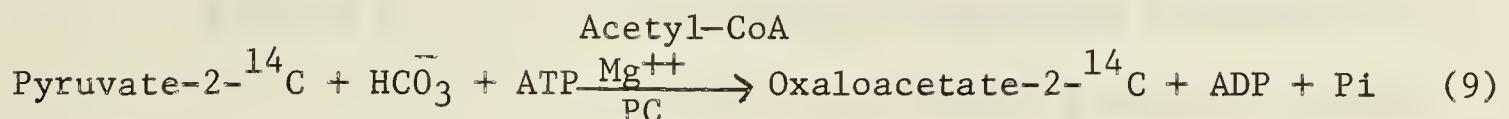


Utter and Keech (1960, 1963) used labelled bicarbonate-¹⁴C and measured the incorporation of ¹⁴C into oxaloacetate and related compounds after releasing the excess of bicarbonate-¹⁴C by acidifying and gassing the mixture with CO₂. However, Utter and Keech (1963) observed that semipurified preparations showed only 50% of the enzyme activity with this method as compared with the spectrophotometric procedure.

Scrutton, Keech and Utter (1965) cautioned that the reaction catalyzed by PC was very complex and the above assay procedures were likely to be subject to considerable interference when crude enzyme preparations were used. They suggested that the exchange reaction between labelled pyruvate and oxaloacetate (final step of the reaction) might be used as a measure of PC activity in crude extracts, as shown in Equation 8.



Although this method is very specific it would involve considerable additional work for separating labelled oxaloacetate from labelled pyruvate and consequently has not been widely adopted. Very recently Bottger *et al.* (1969) have estimated PC activity in rat liver mitochondrial preparations by employing pyruvate- $2-^{14}\text{C}$, coupled with a citrate synthetase trap and an acetyl-CoA regenerating system as shown in Equations 9-11.



The labelled citrate formed was precipitated as the silver salt. The level of radioactivity in the precipitate was used to estimate PC activity. Ballard and Hanson (1967) and Deodhar and Mistry (1969a) have employed a citrate synthetase trap to remove oxaloacetate, while employing bicarbonate- ^{14}C as the primary reagent for measuring PC activity in adipose tissue and liver of rats.

There is some evidence that glutamate oxaloacetate transaminase (GOT) and glutamate may serve as a useful trap system for oxaloacetate and thereby drive the PC catalyzed reaction to completion. Walter, Paetkau and Lardy (1966) reported that addition of glutamate to intact rat liver mitochondria utilizing pyruvate resulted in the formation of aspartate and that the rate of incorporation of bicarbonate- ^{14}C was increased. Somberg and Mehlman (1969) reported increased utilization of pyruvate by guinea-pig liver mitochondria upon the addition of glutamate.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

A series of experiments were conducted during 1967-69 and the results obtained are reported in the following sections:-

Section I - Liver pyruvate carboxylase activity in relation to the biotin status of poultry.

Section II - Influence of season, dietary protein and level of biotin on performance and pyruvate carboxylase activity in livers of poult's.

Section III - Effect of feeding TCA cycle intermediates on the status of biotin in the metabolism of chicks.

GENERAL EXPERIMENTAL

Rearing of Chicks and Poulets

Day old male chicks (Dominant White X White Plymouth Rock) were raised in electrically heated batteries with raised screen floors and day old poulets (Broad Breasted White) were kept in floor pens. Feed and water were supplied ad libitum except where some other treatments are specifically mentioned. Chicks were weighed at weekly intervals and poulets at bi-weekly intervals. The appearance of deficiency symptoms was recorded. In each experiment liver samples were obtained by selecting birds at random from each of the groups.

Preparation of Liver Powder

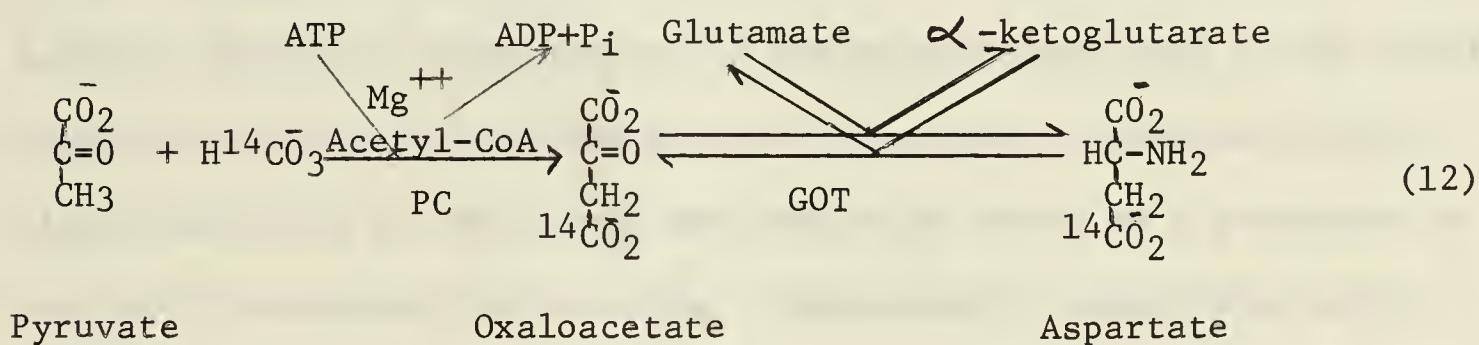
Birds were killed by decapitation and the livers were removed at once. The gall bladders were separated intact from the livers and were discarded. Each liver was then weighed, placed in a polythene bag and immersed in ice.

Liver homogenates were prepared by adding 9 volumes (ml/g) of 40 mM tris-HCl buffer pH 7.8, at 2-5 C to the liver tissue and blending in a Waring blender for 15-20 sec. The homogenate was then strained through a double layer of muslin cloth to remove connective tissue. The strained homogenate was lyophilized at -60 C and 150 μ pressure. The protein content of a sample of strained homogenate and lyophilized powder was determined by the biuret method outlined by Cornall, Bardawill and Davis (1949). Details of the method are given in Appendix I.

Estimation of Pyruvate Carboxylase (PC) Activity of Liver

Pyruvate carboxylase activity of liver was estimated by measuring the incorporation of labelled bicarbonate-¹⁴C into non-acid-volatile form, in the presence of pyruvate, by adapting the procedure of Utter and Keech (1963).

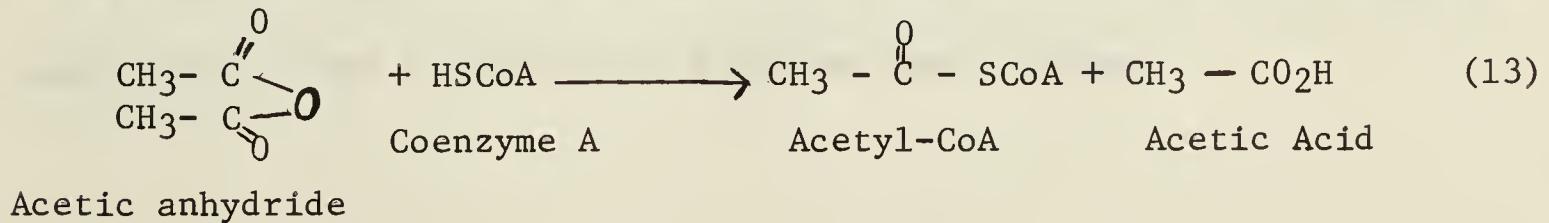
Glutamate and GOT were included in the assay mixture to remove the product of the reaction and Cleland's reagent (Dithiothreitol) was added to protect the sulphhydryl groups of the enzyme. The reactions involved are presented in equation 12.



The unit used to express the PC activity was one μ mole of bicarbonate- ^{14}C incorporated, in 1 minute, into non-acid-volatile form, in the presence of pyruvate at 30 C. Details of the assay procedure and method of calculating PC activity are given in Appendix II.

Preparation of Acetyl-CoA

Acetyl-CoA was prepared from coenzyme A and acetic anhydride by adapting the method of Sly and Stadtman (1963) for formyl-CoA. The reaction is presented in Equation 13.



Details of the procedure are given in Appendix III.

Section I - Liver Pyruvate Carboxylase Activity in Relation to the
Biotin Status of Poultry

INTRODUCTION

As discussed in section C of the "Review of Literature" it has been established that biotin is involved in four enzymes found in mammalian and avian species. There has, however, been no systematic effort to relate the concentration of the enzymes with the biotin status of any species. Because pyruvate carboxylase (PC) is involved with the supply of C₄-dicarboxy acids which can have many direct and indirect metabolic consequences, it was hypothesized that of the biotin-dependent enzymes PC might be the most sensitive to changes in the biotin nutrition of the animal and thus might serve as a parameter of the biotin status of the organism. Consequently, experiments were undertaken to standardize an assay procedure for PC activity in crude liver preparations and to study the effect of biotin level of the ration fed on PC activity of livers of chicks.

Part A. - Standardization of PC Assay

OBJECT

An assay procedure based on the method outlined by Utter and Keech (1963) was modified by the inclusion of a GOT trap and Cleland's reagent in the assay mixture. Before using the method routinely, a series of 6 trials was conducted to standardize the procedure and to verify the usefulness of the modifications that had been adopted.

EXPERIMENTAL AND RESULTS

Trial 1. Effect of time-lapse on specific activity of NaHCO₃-¹⁴C

The results of preliminary trials had indicated that even when using the same volume of stock solutions of labelled and unlabelled NaHCO₃ for preparing premixtures, the specific activity of bicarbonate-¹⁴C varied from day to day. It seemed that an explanation for the variation was the possible loss of CO₂-¹⁴C through exchange with atmospheric CO₂. To test this possibility, a solution containing 125 mM tris-HCl buffer, pH 7.8; 2.5 mM NaHCO₃ and 0.333 μ c of NaHCO₃-¹⁴C per ml was stored in a stoppered tube. Triplicate samples of 0.2 ml were taken by opening the tube briefly, at 10 minute intervals for one hour and were counted by the usual procedure.

The results of the trial (Fig. 3) indicated that at the pH of the assay system there was considerable loss of CO₂-¹⁴C through exchange with atmospheric CO₂. The radioactivity remaining after 60 min was only 90% of the initial value.

For subsequent work the stock solution of labelled bicarbonate was adjusted to pH 9.0 and stored at -10°C. In order to adjust for any loss of CO₂-¹⁴C that might occur during the incubation of a set of assays, samples of the premixture (containing all the reagents other than pyruvate and enzyme extract) were taken just before and immediately after completing the incubation of the set of assays and an average of the radioactivity measured was used in calculating the specific activity of the bicarbonate-¹⁴C.

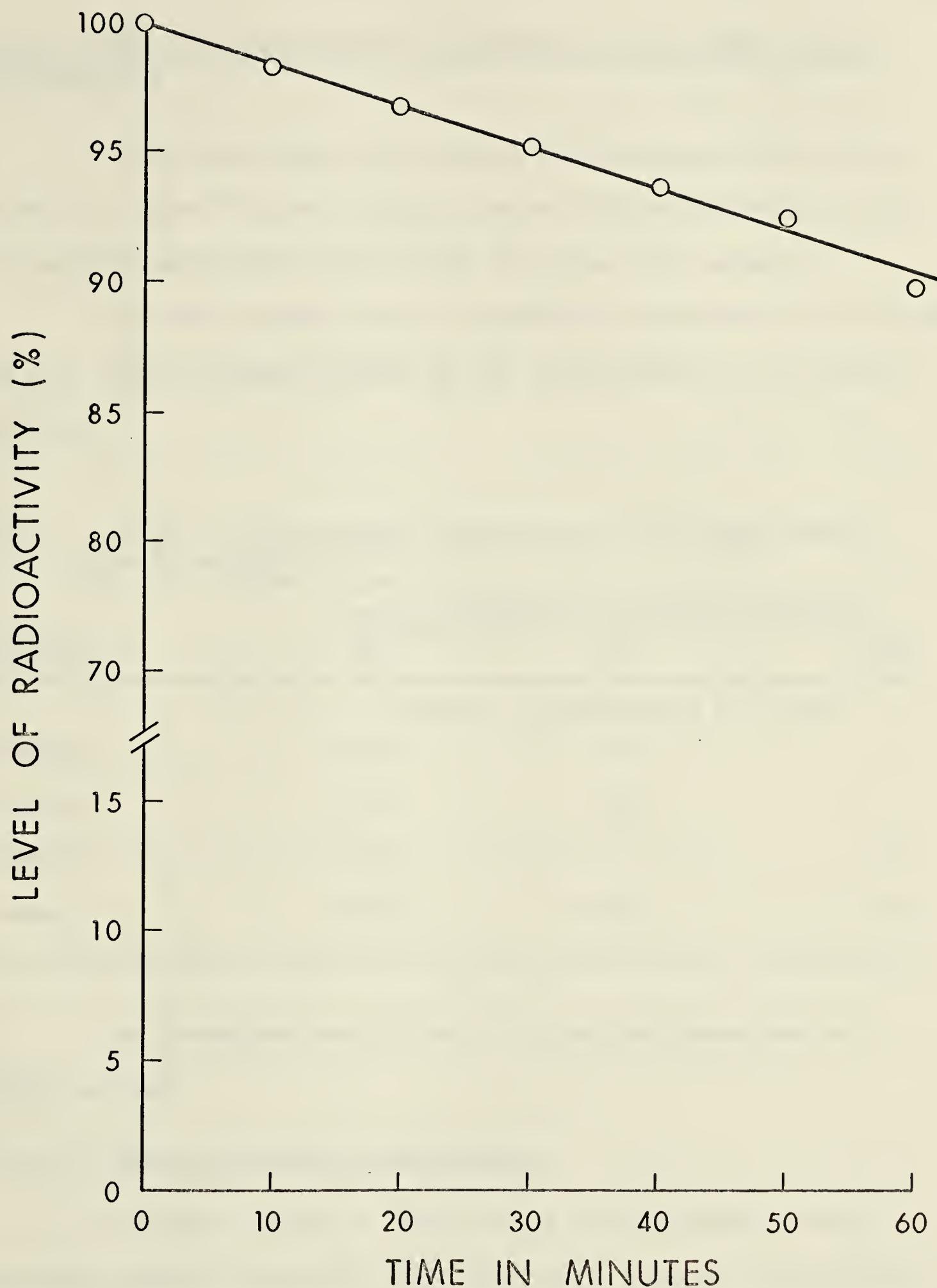


Fig. 3. Loss of radioactivity from sodium bicarbonate- ^{14}C at pH 7.8.

Trial 2. Effect of liver powder concentration in the crude extract on PC activity

Lyophilized chick liver powders were extracted with varying volumes of buffer to give a range of concentrations (20, 40 and 100 mg of powder/ml extractant) and their PC activities were measured.

The data obtained (Table 1) indicated that measured PC activity per g of liver increased markedly as the concentration of the extract increased.

Table 1. Effect of liver powder concentration in the crude extract on PC activity

Age of Chicks	<u>mg of lyophilized powder/ml extractant</u>		
	20	40	100
(units of PC activity/g of liver)			
2 weeks	0.88	3.51	-
3 weeks	3.20	6.04	-
3 weeks	0.76	-	9.92
3 weeks	2.50	4.53	9.52

For subsequent work 100 mg of lyophilized powder per ml of buffer was used.

Trial 3. Effect of diluent on PC activity

Extracts prepared by using 100 mg of liver powder per ml extractant usually contained a high level of PC activity and therefore needed to be diluted for assay. Two diluents, namely 50 mM Tris-HCl buffer, pH 7.6 and albumin solution (100 mg/ml), pH 7.6 were tested for suitability as diluents.

The results of this trial (Table 2) indicated that the albumin solution was a satisfactory diluent because when albumin was used as a diluent no appreciable loss of activity was noted but when tris-HCl buffer was used PC activity decreased to approximately one third of that found in undiluted samples.

Table 2. Effect of diluent on PC activity

Enzyme Source	Age of Birds	Diluent	Dilution	PC Activity	
				Undiluted	Diluted
(Units/g of liver)					
Chick liver	3 wks	Tris-HCl buffer	5X	18.01	5.74
Poult liver	2 days	- do -	5X	7.91	2.52
Chick liver	3 wks	Albumin ¹ solution	5X	18.02	18.81
Poult liver	3 wks	- do -	2X	22.17	21.88
Poult liver	3 wks	- do -	2X	19.81	17.79

¹ Bovine albumin

Trial 4. Effect of enzyme concentration on PC activity

For meaningful interpretation of results, it is necessary that measurements be conducted within the range of linear response in activity to increased enzyme concentration. From time to time, the effect of enzyme concentration on PC activity was checked and the results of four such runs are shown in Fig. 4.

The results indicated that enzyme concentrations yielding up to approximately 150 μ moles of product per 5 min (measured in terms of the incorporation of $\text{HC}O_3^{-14}\text{C}$ into non-volatile form, in the presence of pyruvate at 30 C) was within the linear range. This limit was strictly adhered to in the work included in this report.

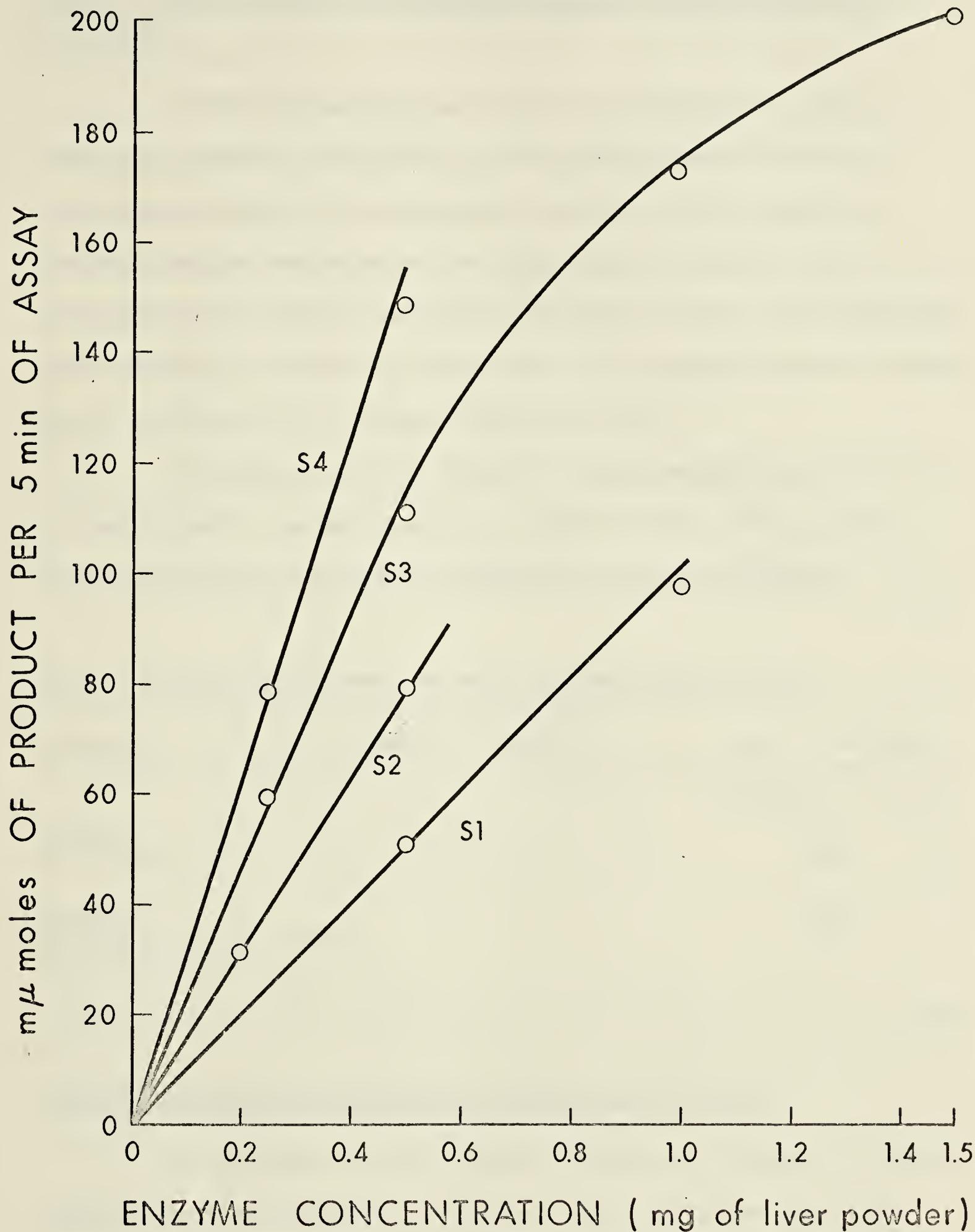


Fig. 4. Effect of enzyme concentration on pyruvate carboxylase activity.

Trial 5. Effect of GOT and Cleland's reagent (Dithiothreitol) on PC activity

A GOT trap was included in the assay mixture in order to remove oxaloacetate, the product of the reaction catalyzed by PC and thereby to assist in driving the reaction towards completion. Cleland's reagent was added to the assay mixture to protect the sulfhydryl groups of PC. In order to determine whether these additions were beneficial, a number of lyophilized liver preparations were assayed for PC activity, with or without these additions.

The results obtained (Table 3) indicated that the addition of GOT and Cleland's reagent resulted in higher levels of PC activity. Consequently their addition was adopted as a routine procedure.

Table 3. Effect of GOT and Cleland's reagent on PC activity

Treatment	Number of Samples	Avg PC Activity (units/g of liver)
Complete	5	31.8
GOT deleted	5	26.6
Complete	3	23.8
Cleland's reagent deleted	3	21.2

Trial 6. Comparison of methods of extracting PC activity

As discussed in the "Review of Literature, Section E" various methods have been suggested for extracting PC from liver and other tissues. Some of the procedures, using fresh tissues are simple and their use would be desirable provided that results were comparable to those obtained by extracting lyophilized tissue powders.

Two samples of liver of two week old chicks were treated as outlined in Table 4 and the PC activities of the preparations were measured. The results obtained (Table 4) showed that the fresh liver homogenates gave very low PC activity and that the treatment with triton X-100 resulted in some increase in yield of PC activity but lyophilization of liver tissue followed by extraction at room temperature gave much higher levels of PC activity. It was therefore decided to adopt lyophilization of liver tissue as a standard procedure.

Table 4. Comparison of methods of extracting PC activity

Enzyme Source ¹	Method of Extraction	PC Activity	
		Sample 1	Sample 2
(units/g of liver)			
Fresh liver	a) Homogenizing at room temperature	1.61	1.41
	b) Above homogenate treated as follows:		
	i) 1 min. with 0.2% triton X-100	2.47	1.98
	ii) 10 min. with 0.2% triton X-100	2.98	1.89
	iii) 1 min. with 0.5% triton X-100	--	2.09
	iv) 3 min. with 0.5% triton X-100	--	2.18
	v) 5 min. with 0.5% triton X-100	--	2.18
Lyophilized liver powder	c) Homogenizing at room temperature	20.61	16.51

¹ Samples from the same liver were used for preparing fresh liver homogenate and lyophilized liver powder.

DISCUSSION

The loss of radioactivity that occurred because of exchange between bicarbonate-¹⁴C and atmospheric CO₂ with lapse of time was sufficiently high to necessitate the use of a corrected value for specific

activity of bicarbonate. In this study, involving comparative work completed within a short time, samples were taken just before and immediately after completing the incubation of a set of assays and the average value of their radioactivities was used to calculate the specific activity of bicarbonate. For very precise work involving labelled bicarbonate as a primary reagent, a time-loss curve could be constructed and used for estimating specific activity at a given time.

The results of trials 2 and 3 suggest that PC is a hydrophobic enzyme because, the PC activity increased markedly with an increase in the concentration of the enzyme extracts and concentrated albumin solution proved to be a good diluent whereas dilution with buffer resulted in a considerable loss of PC activity. Some evidence from other laboratories also support this conclusion. Scrutton and Utter (1965) reported that high salt concentration was required to stabilize the semipurified and purified preparations of PC and Bottger et al. (1969) reported that the use of 50% glycerol solution protected rat liver PC against cold inactivation.

The expression of PC activity can be enhanced by immediately removing oxaloacetate from the site of the reaction. In this study the inclusion of a GOT trap substantially increased the fixation of bicarbonate-¹⁴C. Earlier Walter et al. (1966) and Somberg and Mehlman (1969) reported that the addition of glutamate to intact mitochondria utilizing pyruvate resulted in the formation of considerable amounts of aspartate and increased carboxylation of pyruvate. Ballard and Hanson (1967), Bottger et al. (1969) and Deodhar and Mistry (1969a) have employed a citrate synthetase trap for estimating PC activity but did not report any comparisons with assays in which the trap was not used.

PC seems to have very sensitive sulfhydryl groups which are important for its activity. Keech and Utter (1963) reported that PC was inactivated by inhibitors of sulfhydryl groups. In this study Cleland's reagent which keeps the sulfhydryl groups in the reduced form, tended to increase PC activity of crude extracts of chick liver.

Contrary to the assertion of Wagle (1964) the results of trial 6 showed that simply homogenizing the liver at room temperature was not sufficient to release the entire PC activity. Ballard and Hanson (1967) have reported similar results with rat adipose tissue mitochondria. The probable reason for very low PC activity exhibited by the intact mitochondria of rat liver (Wagle, 1964; Freedman and Kohn, 1964) and by chick liver homogenates containing mitochondria (Trial 6) could be that some essential factors such as acetyl-CoA or ATP failed to cross the mitochondrial membrane and were not synthesized in the intact mitochondria because of the anaerobic conditions in the assay system.

SUMMARY

1. An assay method for estimating PC activity of crude extracts of avian liver by measuring the incorporation of bicarbonate-¹⁴C into non-acid-volatile form, was modified by including a GOT trap to remove oxaloacetate and Cleland's reagent to protect the sulfhydryl groups of the enzyme. Both modifications resulted in an increase in PC activity as compared to assays conducted without the modifications.

2. A considerable loss of radioactivity from bicarbonate-¹⁴C kept at pH 7.8 occurred because of a rapid exchange with atmospheric CO₂. An adjustment in the specific activity of bicarbonate-¹⁴C was necessary.

3. A high concentration of liver extracts was essential to keep PC in active form. Extracts of 100 mg lyophilized powder of liver per ml of buffer were stable and could be diluted up to 5 fold in a solution of 100 mg of bovine albumin/ml, pH 7.6. Dilution in 50 mM tris-HCl buffer, pH 7.6 resulted in decreased PC activity.

4. Lyophilization of liver resulted in extraction of high levels of PC activity. Homogenizing fresh liver at room temperature or treating the homogenate with triton X-100 gave only a small fraction of the activity.

Part B. - Effects of dietary biotin level on growth and the PC activity of chick liver

OBJECT

The purpose of this study was to relate the PC activity of the liver with the biotin nutrition of chicks. Two experiments were designed to study the effects of age of chicks and biotin level in the ration fed on growth, occurrence of deficiency symptoms and pyruvate carboxylase levels in the chick liver.

EXPERIMENTAL

In the first experiment two groups of 20, day-old male chicks were fed a basal ration (Table 5) supplemented with either 0 or 320 μ g of d-biotin per kg. Feed and water were supplied ad libutum.

Four chicks from each group were sacrificed at 9, 16 and 29 days of age and their livers were taken for the determination of PC activity. Individual livers were used to make lyophilized powders and duplicate assays for PC activity were conducted on each preparation.

Table 5. Composition of purified basal ration deficient in biotin.¹

<u>Ingredients</u>	<u>% of ration</u>
Starch	60.70
Corn oil	1.00
Stabilized Tallow	4.00
Vitamin-test casein	18.00
Gelatin	10.00
DL-Methionine	0.10
Mineral Mixture ²	1.22
Manganese sulphate	0.03
Calcium carbonate	1.50
Dicalcium phosphate 2H ₂ O	2.15
Sodium Chloride	0.60
Vitamin Mixture ³	0.35
Choline chloride	0.15
Folic acid (30 mg/g)	0.10
Ascorbic acid	0.10
Total	100.00

¹ Ration contained 25.66% protein and supplied 16 kcal M.E./g of protein.

² Mineral mixture supplied the following levels per kg of ration:
 Potassium dihydrogen phosphate, 9.36 g; Magnesium sulphate, 2.42 g;
 Potassium iodide, 2.9 mg; Ferrous sulphate (FeSO₄.7H₂O), 278 mg;
 Zinc carbonate, 115 mg; Cobalt chloride, 1.7 mg; Sodium molybdate
 (Na₂MoO₄.2H₂O), 8.3 mg; Sodium selenite, 0.22 mg and Copper
 sulphate (CuSO₄.5H₂O), 15.6 mg.

³ Vitamin mixture supplied the following levels per kg of ration:
 Vitamin A, 4,063 IU; Vitamin D₃, 497 ICU, Vitamin E, 120 IU;
 Vitamin K, 2 mg; Thiamine hydrochloride, 3 mg; Riboflavin, 6 mg;
 Calcium pantothenate, 20 mg; Niacin, 50 mg; Pyridoxine, 6 mg;
 Vitamin B₁₂, 0.02 mg; β -aminobenzoic acid, 2 mg and Inositol,
 100 mg.

In the second experiment 72, day-old male chicks were placed on the biotin deficient basal ration for one week. At one week of age the chicks were divided into 6 groups of 10 chicks and fed the basal ration supplemented with 0, 20, 40, 80, 160 or 320 μ g of d-biotin per kg of ration. At 23 days of age all of the surviving chicks were sacrificed and their livers were removed for PC assay. The livers from each group were pooled and lyophilized. Duplicate estimations of PC activity were conducted on each preparation.

RESULTS

Experiment 1

A deficiency of biotin in the ration fed had little effect on the growth rate of chicks up to 9 days of age but subsequently the rate of growth was greatly reduced as compared to the chicks receiving an adequate level of biotin.

Deficiency symptoms similar to those described by Ringrose et al. (1931) appeared in the group fed the biotin-deficient ration. By 3 weeks of age most of the chicks showed evidence of dermatitis and by 4 weeks of age the symptoms became severe. The dermatitis noted included severe lesions on the feet, typical of a biotin deficiency, and encrustations at beak angles and around the eye, however, no evidence of perosis was noted.

A perusal of the data (Table 6) revealed that up to 16 days of age the weight of liver was proportional to the body weight in both groups of chicks but at 29 days of age liver weight was comparatively higher in chicks fed the biotin-deficient ration than the controls fed biotin. Liver protein was apparently not influenced by a deficiency of biotin.

Table 6. Effect of age and biotin-deficiency on growth and liver characteristics of chicks

Ration	Level of d-biotin (μ g/kg)	Age of chicks (days)	Avg body wt (g)	Avg liver wt (g)	Liver Protein (%)	PC activity of liver ¹ (units/g)
Biotin-deficient	0	9	67	2.3	23.5	3.54 \pm 0.51
		16	99	3.1	28.8	2.62 \pm 0.48
		29	161	7.1	24.9	0.45 \pm 0.13
Complete	320	9	69	2.4	24.7	5.67 \pm 1.84
		16	153	4.9	27.7	21.66 \pm 3.14
		29	348	8.8	24.6	16.03 \pm 2.30

¹ Values are means \pm SEM of 4 samples in duplicate.

The effect of age and nutritional treatments on the PC activity of the livers of chicks are given in Table 6 and presented graphically in Fig. 5. In chicks fed the biotin deficient ration, PC activity in the liver continued to decline throughout the experimental period. By 29 days of age the PC activity was only 0.45 units/g of liver. In chicks fed biotin the PC activity increased rapidly and showed a maximum level at 16 days of age (21.7 units/g of liver), followed by a slight decline. The results of this experiment suggest that chicks may be depleted of the biotin carried over in the embryo by feeding a biotin-deficient ration for one week without any harmful effect and that about three weeks of age would be an appropriate time for studying the relationship between biotin supply in the ration and PC activity of chick liver.

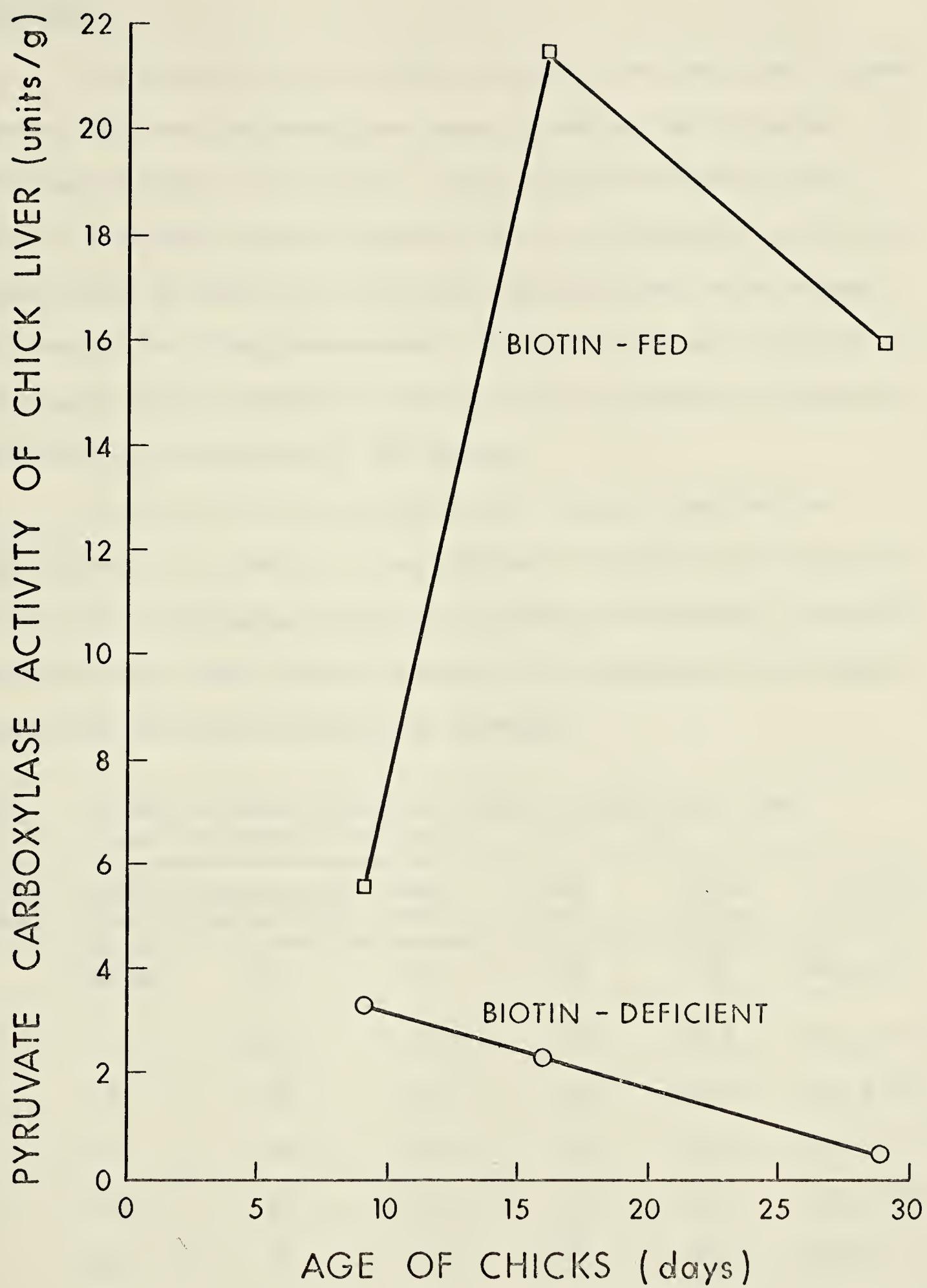


Fig. 5. Effect of age and biotin deficiency on pyruvate carboxylase activity of chick liver.

Experiment 2

The results obtained (Table 7) showed that the rate of growth observed when the biotin-deficient basal ration was fed throughout the experiment was very low (Group 1). The inclusion of biotin in the ration at one week of age at levels of 20, 40, or 80 µg/kg resulted in some increase in growth rate but little difference was noted between the three levels of supplementation. Addition of 160 and 320 µg of biotin/kg of ration resulted in increased rate of growth as compared to the lower levels of biotin in the rations.

Lack of biotin in the basal diet (Group 1) resulted in a 100% incidence of dermatitis. The addition of biotin to the ration at 20, 40, 80, or 160 µg/kg reduced the incidence of dermatitis to 90, 70, 22 and 0% respectively and the severity of the symptoms noted tended to decrease as the level of biotin was increased.

Table 7. Effect of biotin level of ration on growth and liver characteristics of chicks

Group Number	Level of d-biotin (µg/kg)	Incidence of dermatitis ¹ (%)	Avg body wt (g)	Avg liver wt (g)	Liver Protein (%)	PC activity ² of liver (units/g)
1	0	100	109	3.8	28.8	0.71 ± 0.12
2	20	90	172	5.8	28.8	1.56 ± 0.33
3	40	70	168	5.9	31.5	1.32 ± 0.05
4	80	22	168	5.2	32.4	1.96 ± 0.22
5	160	0	213	6.5	32.2	7.58 ± 0.42
6	320	0	232	7.1	30.6	18.18 ± 0.35

¹

Two chicks of group 1 and one from each of groups 4 and 5 died during the experimental period.

² Values are average ± deviation of duplicate estimations on the pooled liver preparation.

Percentage of protein in the livers of chicks was very slightly increased by increasing levels of biotin supply in the rations.

The PC activity in the livers of chicks fed the biotin-deficient ration was very low (0.71 units/g of liver). When 20 μ g of biotin/kg of ration was added a doubling in PC activity was noted but no further increase in PC activity was observed when the biotin level was increased to 40 or 80 μ g/kg of ration. Further increases in biotin levels (160 and 320 μ g/kg) resulted in an appreciable and linear increase in PC activity, as shown in Fig. 6.

DISCUSSION

The results of this study substantiate the earlier inference that the curative dose for biotin deficiency symptoms in chicks may be about 100 μ g of biotin/kg of ration (Ansbacher and Landy, 1941 and Hegsted *et al.*, 1942) and further suggest that biotin requirement for maximal growth of chicks is much higher than the amount required to prevent the occurrence of deficiency symptoms.

The results obtained also suggest that in chicks, biotin usage may have priorities for alternate functions depending upon the extent of its availability. For example, when biotin supply is very limited, most of it may be used for the synthesis of pyruvate holocarboxylase needed to sustain life; when a little more biotin is available, other biotin-dependent enzymes may be enhanced to eliminate the metabolic disorders which result in visible deficiency symptoms and when still higher levels of biotin are available it may then be used to activate more PC to supply essential metabolites required for growth and normal metabolic activity of the body. The sequence

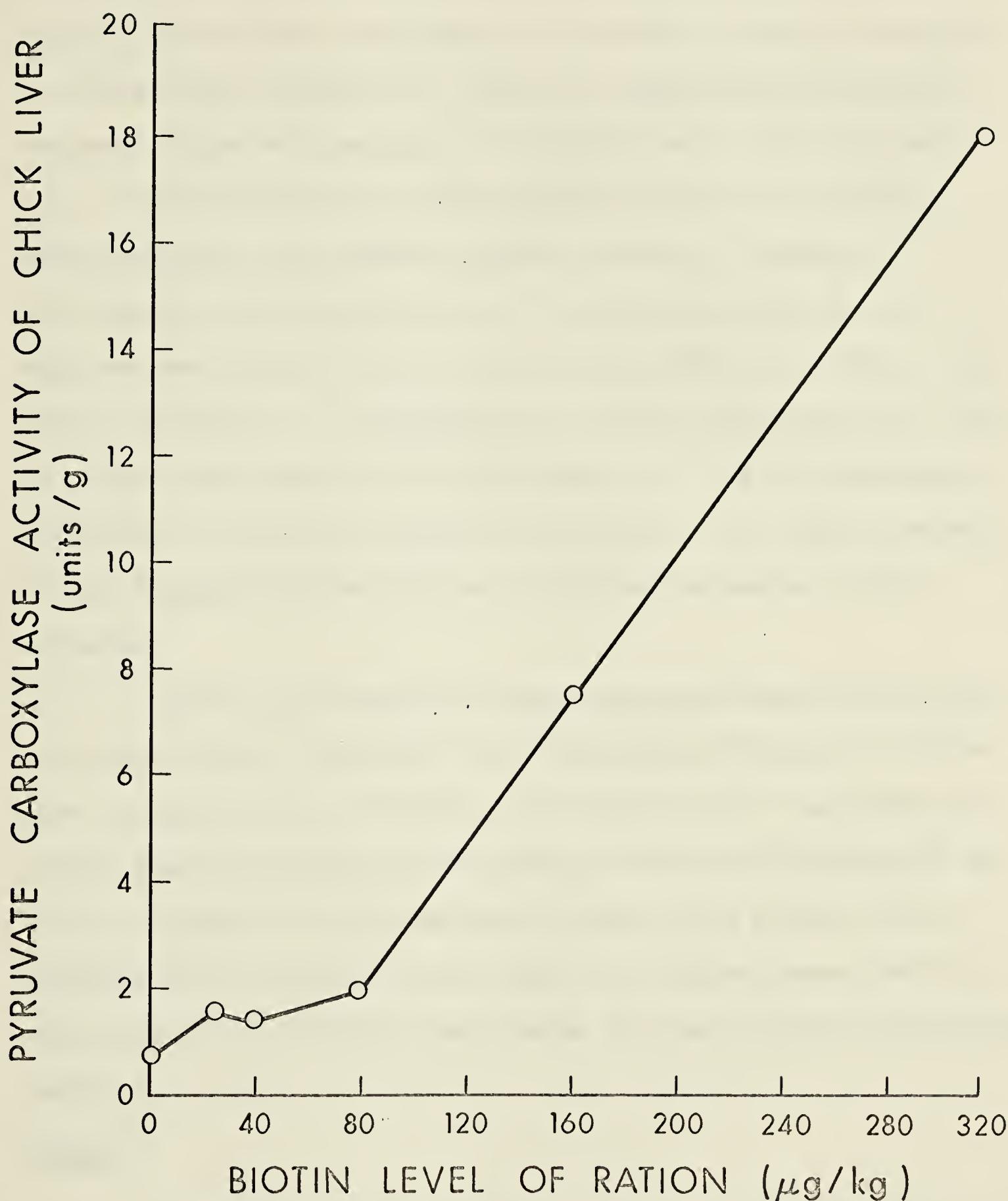


Fig. 6. Effect of biotin level on pyruvate carboxylase activity in livers of chicks at 23 days of age.

of appearance of biotin-deficiency symptoms in chicks fed a biotin-deficient ration also tend to substantiate the above hypothesis, because in such chicks a decrease in PC activity of liver was apparent at 9 days of age, followed by a decrease in weight gain noticeable at two weeks of age and appearance of dermatitis during the third week of age. It may be inferred that the dermatitis noted was a secondary effect resulting from impaired metabolic activity. Evidence of preferential utilization of biotin for specific functions in microorganisms was reported by Potter and Elvehjem (1948), who observed that biotin requirement of L. arabinosus for aspartate synthesis was at least 10 times greater than that for other functions. Thus the preferential utilization of biotin for alternate functions may be a common phenomena in many organisms and may serve as an important metabolic control mechanism.

Another interesting and very significant observation was that the level of biotin supply over the range of 80 to 320 $\mu\text{g}/\text{kg}$ of ration gave a linear increase in PC activity of chick liver. Thus under sub-optimal supply of biotin the PC activity of chick liver represented the status of metabolically active biotin and this could be used as an indicator of the effective biotin supply in a ration whereas the microbial assays of the feedstuffs have failed to do so (Review of Literature, Section B).

SUMMARY

The effects of age and biotin supply on the growth, appearance of deficiency symptoms, liver protein and PC activity of chick liver were studied. The following results were obtained:-

1. Groups of chicks fed biotin-deficient purified ration showed typical symptoms of a biotin deficiency and had very low PC activity in the liver. The addition of an adequate amount of biotin to chick rations alleviated the biotin deficiency symptoms, increased the rate of growth and enhanced the PC activity of liver appreciably.

2. PC activity in livers of chicks fed a biotin-deficient ration showed a linear decline with increase in age. In livers of normal chicks fed biotin, PC activity showed a large increase at about two weeks of age but PC activity declined slightly by four weeks of age.

3. At 23 days of age, the PC activity in the livers of chicks fed a biotin deficient ration was very low (0.71 units/g of liver). When 20 μ g of biotin/kg of ration was added a doubling in PC activity was noted but no further increase in PC was noted when the biotin level was increased to 40 or 80 μ g/kg of ration. Further increase in biotin levels (160 or 320 μ g/kg) resulted in an appreciable and linear increase in PC activity. It is suggested that at about three weeks of age the PC activity of chick liver may be used to assess the biologically effective supply of biotin in the rations.

4. The effects of supplying graded levels of biotin in the ration and the pattern of the appearance of deficiency symptoms suggested that in chicks biotin usage for different functions may have priorities depending upon its supply. This hypothesis has been discussed.

Section II. Influence of Season, Dietary Protein and Level of Biotin on Performance and Pyruvate Carboxylase Activity in Livers of Poult

INTRODUCTION

The occurrence of a syndrome resembling a biotin deficiency, has been reported in turkey poult fed practical rations (Review of Literature, Section B). It has been noted that incidence of the disorder was increased when rations containing meat meal and fish meal as the only source of supplementary protein were fed as compared to rations containing soybean meal (Robblee, unpublished data). It has also been noted that the incidence and severity of the disorder increased as the hatching season progressed. Consequently an experiment was designed to study the effect of protein source, biotin level of the ration and season on the occurrence of the disorder and on the PC activity in liver.

EXPERIMENTAL

Two trials were conducted using poult hatched on Feb. 18th, 1969 in the first trial and on April 15th, 1969 in the second.

In each trial the poult available for the experiments were divided into six comparable groups (62 poult/group in Trial 1 and 17 poult/group in Trial 2) and fed the rations shown in Table 8. The poult were weighed at two and four weeks of age and at the time of weighing the poult were examined for symptoms of the syndrome including hock disorder, dermatitis and broken feathers.

At three weeks of age two groups of four poult each were selected at random from each ration treatment. The livers of these groups of poult were pooled and lyophilized. The resulting livers powders were then assayed, in duplicate, for PC activity.

Table 8. Composition of turkey starter rations

Ingredients	Ration Number					
	1	2	3	4	5	6
	(%)	(%)	(%)	(%)	(%)	(%)
Ground corn	10	10	10	10	10	10
Ground wheat	46	46	38	38	32	32
Wheat shorts	9.215	9.215	1.715	1.715	0.715	0.715
Stabilized animal fat	-	-	2	2	3	3
Dehydrated alfalfa meal	-	-	2	2	2	2
Meat meal (55% protein)	22	22	10	10	-	-
Herring meal (72% protein)	10	10	5	5	7	7
Soybean meal (44% protein)	-	-	28	28	40	40
Iodized salt	0.25	0.25	0.25	0.25	0.25	0.25
Ground lime-stone	1	1	1	1	1.5	1.5
Dicalcium phosphate (18.5% Ca and 20.5% P)	-	-	0.5	0.5	2	2
Manganese sulphate	0.025	0.025	0.025	0.025	0.025	0.025
Zinc oxide	0.01	0.01	0.01	0.01	0.01	0.01
Micronutrient Mix ¹	1.5	1.5	1.5	1.5	1.5	1.5
Biotin ²	-	+	-	+	-	+

Calculated analyses:-

Metabolizable energy (kcal/kg)	2649	2649	2697	2697	2689	2689
Protein %	27.7	27.7	27.9	27.9	28.2	28.2
Biotin content (μg/kg)	164	384	216	436	249	469

¹ Micronutrient Mix supplied the following levels per kg of ration:-

Vitamin A, 5940 IU; Vitamin D₃, 1650 ICU; Vitamin E, 22 IU; menadione sodium bisulphate, 1.1 mg; riboflavin, 3.3 mg; calcium pantothenate, 11 mg; niacin, 22 mg; choline chloride, 187 mg; Vitamin B₁₂, 0.0066 mg; folic acid, 3.3 mg; penicillin, 4.4 mg; DL-methionine, 500 mg.

² Biotin additions were made at the level of 220 μg/kg.

RESULTS

A perusal of the data (Table 9) showed that the rate of growth of poult was affected by the source of protein supplement but neither season nor the addition of biotin to various rations had any appreciable effect on growth performance of poult. When meat meal and fish meal were the only source of protein supplement (Groups 1, 2, 7 and 8) rate of growth was low. The use of soybean meal to replace 55% of meat meal and 50% of fish meal (Groups 3, 4, 9 and 10) improved the rate of growth very markedly. When soybean meal was the principal protein supplement (Groups 5, 6, 11 and 12), growth rate was further improved. In one instance, the addition of biotin gave an appreciable increase in the rate of growth and increased the body weight from 519 g (Group 3) to 597 g (Group 4). In all the other combinations the weight of poult in biotin supplemented and control groups was similar. Liver weight was directly related to body weight.

Typical symptoms of the syndrome were observed in poult fed rations supplemented with meat meal and fish meal only and the syndrome was largely prevented by adding biotin to the ration. Among the poult fed this ration without added biotin (Groups 3 and 9) 50-67% showed the appearance of hock disorder, dermatitis and broken feathers whereas when the same ration was supplemented with 220 μ g of biotin/kg (Groups 4 and 10) the incidence of various symptoms was reduced to 2-15% and the severity of the symptoms was considerably lessened.

The pyruvate carboxylase activity of poult liver was affected by the source of protein, biotin level of the rations and season. The PC activity in the liver of poult fed meat-fish meal supplemented ration (Groups 1 and 7) was much lower than those of other groups.

Table 9. Effect of protein source and biotin supply on performance and liver PC activity of poult

Trial No.	Group No.	Protein Supplement	Biotin Supply-	Observations at 4 wk of age				Properties of liver at 3 wk of age			
				Avg Body wt	Hock Disorder	Derma-titis	Broken feather	Avg Wt	Protein	PC activity ¹	PC activity ²
1	1.	Meat-Fish Meal	-	4.24	54	59	7.1	27.3	10.16±2.35	----	----
2.	2.	Meat-Fish Meal	+	4.49	2	15	8	7.2	25.8	17.72±0.08	7.56
3.	3.	Meat-Fish Soybean Meal	-	5.19	0	13	0	9.7	25.3	18.44±1.65	----
4.	4.	Meat-Fish Soybean Meal	+	5.97	2	8	0	8.4	25.6	23.65±2.95	5.21
5.	5.	Soybean Meal	-	6.23	2	8	9	9.7	26.3	17.82±0.97	----
6.	6.	Soybean Meal	+	6.13	0	4	4	11.0	25.8	20.99±1.18	3.17
2	7.	As 1	-	3.90	67	50	67	6.2	24.9	9.60±1.43	----
8.	8.	As 2	+	4.05	0	12	0	8.8	24.8	17.50±3.52	7.90
9.	9.	As 3	-	5.92	0	0	0	9.6	24.7	12.40±0.37	----
10.	10.	As 4	+	5.87	0	0	0	8.9	25.5	18.81±0.86	6.41
11.	11.	As 5	-	6.24	0	0	0	9.6	26.8	13.47±0.03	----
12.	12.	As 6	+	6.04	14 ^a	0	0	10.1	27.5	15.58±2.01	2.01

1 Values are average ± deviations of the values of two preparations from the average.

2 Increase in PC activity in response to supplemental biotin (220 µg/kg of ration).

a The high percentage indicated may not be real because in this group only one poult was showing slight hock disorder but the number of poult in each of groups 7-12 was very small and calculated % became very high.

Addition of biotin to this ration (Groups 2 and 8) resulted in a doubling of PC activity, to a level comparable to those of poult fed rations supplemented with soybean meal (Groups 3, 5, 9 and 11). Addition of biotin to rations supplemented with soybean meal resulted in a small increase in PC activity of poult liver as compared to the ration containing soybean meal without biotin (Groups 4, 6, and 12 vs. Groups 3, 5, 9 and 11). The maximum amount of PC activity observed was 23.65 units/g of liver in poult of group 4.

The effect of season on PC activity in livers of poult was variable. The PC activity in livers of poult fed soybean meal containing rations was about 25-33% lower in Trial 2 (Groups 9, 10, 11 and 12) than in Trial 1 (Groups 2, 4, 5 and 6). No effect of season was noted in poult fed rations without soybean meal (Groups 1 and 2 of Trial 1 and Groups 7 and 8 of Trial 2).

Protein content of poult liver was not affected by the source of protein supplement, biotin level of the rations or season.

DISCUSSION

It seems that the available biotin content of meat-fish meal supplemented ration was just marginal because in the groups fed this ration (Groups 1 and 7) 50-67% of poult developed typical symptoms of biotin deficiency, had a low rate of growth and showed low PC activity in livers. Biotin supplementation of this ration resulted in a two fold increase in PC activity and prevented the occurrence of deficiency symptoms but failed to improve rate of growth. The biotin supplemented ration contained 384 μ g of biotin/kg, which is much more than the suggested level of 200-300 μ g of biotin/kg of ration, for maximal growth of turkey poult (Jensen, 1969). Also in this experiment groups fed

rations containing soybean meat but without supplemental biotin, grew rapidly although the calculated biotin content of the rations fed was only 216 $\mu\text{g}/\text{kg}$ (Groups 3 and 9) and 249 $\mu\text{g}/\text{kg}$ (Groups 5 and 11). Thus it may be concluded that either the fish-meat meal supplement lacked some growth factor (other than biotin) which is present in soybean meal or the use of meat-fish meal supplement resulted in some imbalance of nutrients and consequently reduced growth rate of turkey poult.

Examination of the data (Table 9) showed that the effect of season of hatch on the PC activity in livers of the poult was variable and seemed to be related to the source of protein supplement used in the ration. It was also noted that increase in PC activity in the poult liver, resulting from a biotin supplement of 220 $\mu\text{g}/\text{kg}$ of ration, was inversely proportional to the amount of soybean meal in the rations. These observations tend to suggest that effective biotin supply of rations containing soybean meal showed a decline with lapse of time or perhaps some factor carried in soybean meal was responsible for the destruction of biotin. However the possibility exists that with the advance of the hatching season, a change in the microflora of the intestine occurred which reduced the synthesis of biotin in the intestinal tract and may have reduced the total amount of biotin available for absorption. Recently it has been reported that microbial assays for biotin gave very variable recoveries of added biotin to turkey rations (Jensen and Martinson, 1969) and that the inclusion of high levels of soybean meal in the rations resulted in feces adherence and an associated dermatitis on the bottom of the feet of poult. These reports suggest the destruction of available biotin from the mixed rations and the probable association between the factor involved in such a destruction and soybean meal.

SUMMARY

The effects of the source of protein supplement, biotin level in turkey starter ration and hatching season were investigated and the following results were obtained:-

1. When meat meal and fish meal were the only source of protein supplement, the ration was marginal in biotin supply; 50-67% of the poult fed this ration developed symptoms of a biotin deficiency, grew at a slow rate and had low PC activity in livers. The supplementation of this ration with biotin prevented the deficiency symptoms, resulted in a two fold increase in PC activity in livers of poult but failed to improve the rate of growth.

2. When soybean meal supplied about one half or almost all the supplemental protein, the poult were free from any signs of the syndrome, grew rapidly and had much higher PC activity in livers than poult fed meat-fish meal protein supplement. Addition of biotin to rations in which soybean meal supplied about one half of the protein supplement, increased the PC activity in livers of poult by 33% in an early hatch and by 50% in a late hatch. The corresponding increase when soybean meal supplied almost all the protein supplement were 18% and 16% respectively.

3. Progression of the hatching season did not show any effect on PC activity in livers of poult fed rations containing meat-fish meal protein supplement but when soybean meal was included in the rations the PC activity of livers of poult of a late hatch was 33-50% lower than that of poult from an early hatch.

Section III. Effects of Feeding TCA Cycle Intermediates on the Status of Biotin in the Metabolism of Chicks

INTRODUCTION

As discussed in section 1, part B, there were some indications that in chick liver, biotin usage may have priorities for alternate functions depending upon the extent of its availability. It was also found that the use of 160 μ g of biotin/kg of ration, completely prevented the occurrence of biotin deficiency symptoms in chicks but much higher levels of biotin were required for optimal growth and maximal PC activity in liver. It was reasoned that inclusion of some precursors of oxaloacetate in the ration might reduce the dependence of the organism on PC and consequently the metabolic activity and growth might be improved if a medium level of biotin were available. In support of this view, Dakshinamurti and Mistry (1963) reported that on feeding rations containing 10% sodium succinate to biotin-deficient rats and chicks, the incorporation of labelled amino acids into various subcellular fractions of liver was restored to the level of animals fed biotin. It was therefore hypothesized that detailed studies along this line could be valuable in understanding metabolic lesions which result in symptoms of a biotin deficiency.

As all of the TCA cycle intermediates can be transformed into oxaloacetate without involving biotin dependent enzymes, studies were conducted to examine the effect of including some of these compounds in the rations on the status of biotin in the metabolism of chicks. A preliminary experiment was conducted to develop a suitable basal ration for the studies.

Experiment 1. Developing a low protein ration

OBJECT

The object of this experiment was to develop a suitable basal ration for studying the effect of TCA cycle intermediates on the metabolic status of biotin in chicks.

It was reasoned that for studies of this sort the supply of unspecified precursors of oxaloacetate in the basal ration should be kept as low as possible. In order to accomplish this, it was necessary that the protein in the ration be well balanced in relation to the requirement of the chick and that the calorie:protein ratio be slightly higher than the optimum ratio. In poultry, synthesis of protein is considered to be proportional to the most limiting essential amino acid in the diet with excess amounts of other amino acids being degraded. Some of the amino acids, namely aspartic acid, glutamic acid, proline and histidine may serve as potential sources of oxaloacetate without involving PC, but some of the other amino acids require biotin-dependent enzymes for their catabolic utilization. Thus it was considered desirable to reduce the excess of each amino acid to the minimum possible.

EXPERIMENTAL

Three trials were conducted in succession and a low protein ration well balanced in essential amino acids (EAA) was developed.

For Trial 1, pre-mixtures 1-3 (Table 10) were used to formulate experimental rations 1, 2 and 3 respectively (Table 11). Each of the pre-mixtures contained EAA in the proportion suggested by Dean and Scott (1965) which was considered to produce optimum balance of amino acids for growing chicks. Pre-mixture 1 contained 14 g of casein protein and a mixture of limiting EAA to bring each EAA in the right

Table 10. Composition of pre-mixtures - Section III

	Pre-mixture number			
	1	2	3	4
(a) <u>Constant ingredients</u>	(g)	(g)	(g)	(g)
Mineral mixture ¹			1.25	
Calcium carbonate			1.50	
Dicalcium phosphate (feed grade)			2.15	
Vitamin mixture ¹			0.70	
Choline chloride			0.20	
Folic acid (30 mg/g)			0.10	
Ascorbic acid			<u>0.10</u>	
			<u>6.00</u>	
(b) <u>Variable ingredients</u>				
Vitamin-test				
casein ²	16.12	14.50	--	14.77
Amino acid Mix. I ³	2.96	2.66	--	2.66
Amino acid Mix. II ³	--	--	13.65	--
L-glutamic acid	0.65	3.41	9.60	--
Ammonium bicarbonate	--	--	--	1.76
Sodium chloride	0.60	0.60	--	0.88
Sodium bicarbonate	--	--	1.00	--
Aluminum hydroxide	--	--	0.60	--
Corn starch	<u>5.67</u>	<u>4.83</u>	<u>1.15</u>	<u>1.93</u>
Total	32.00	32.00	32.00	32.00

1 Amount of each mineral and each vitamin supplied in the pre-mixtures is given for the complete rations in Table 11, foot note 1.

2 Casein supplied 14 g, 12.6 g and 12.6 g of protein in pre-mixtures 1, 2 and 4 respectively, based on the nitrogen content of individual lots of casein used.

3 Composition of amino acid mixtures used are given in Appendix IV.

Table 11. Composition of rations - Section III

Ingredients ¹	Ration Number								
	1	1a	1b	1c	1d	2	2a	3	4
Pre-mixture 1 (g)	32	32	32	32	32	-	-	-	-
" " 2 (g)	-	-	-	-	-	32	32	-	-
" " 3 (g)	-	-	-	-	-	-	-	32	-
" " 4 (g)	-	-	-	-	-	-	-	-	32
Corn starch (g)	41	51	61	55	50	41	57	41	57
Cellulose (g)	3	3	3	3	3	3	7	3	7
Corn oil (g)	4	4	4	10	15	4	1	4	1
Stabilized tallow (g)	-	-	-	-	-	-	3	-	3
Biotin ²	+	+	+	+	+	+	+	+	-
Total (g)	80	90	100	100	100	80	100	80	100

Calculated Analyses:-

Protein

(g in total) 17.6 17.6 17.6 17.6 17.6 17.6 17.6 17.6 17.6 17.6

Metabolizable

energy

(kcal/g of ration) 3.93 3.95 3.96 4.25 4.49 3.94 3.71 3.90 3.71

Cal: Protein

(kcal M.E./g of protein) 17.9 20.2 22.5 24.1 25.5 17.9 21.1 17.7 21.1

¹ The pre-mixtures used supplied the following:-

(a) Minerals - Manganese sulphate, 30 mg; Potassium dihydrogen phosphate, 136 mg; Magnesium sulphate, 242 mg; Potassium iodide, 0.20 mg; Ferrous sulphate ($FeSO_4 \cdot 7H_2O$), 27.8 mg; Zinc carbonate, 11.5 mg; Cobalt chloride, 0.17 mg; Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), 0.83 mg; Sodium selenite, 0.022 mg and Copper sulphate ($CuSO_4 \cdot 5H_2O$), 1.56 mg.

(b) Vitamins - Vitamin A, 610 IU; Vitamin D, 74 ICU; Vitamin E, 18 IU; Vitamin K, 0.3 mg; Thiamine hydrochloride, 0.45 mg; Riboflavin, 0.9 mg; Calcium pantothenate, 3 mg; Niacine, 7.5 mg; Pyridoxine, 0.9 mg; Vitamin B₁₂, 2 µg; β -aminobenzoic acid, 0.3 mg and Inositol, 15 mg.

²

32 µg of d-biotin was supplied in each case.

proportion. Pre-mixture 2 was made from the same ingredients but contained each EAA at 90% of the level present in pre-mixture 1 and pre-mixture 3 supplied each EAA at the same level as pre-mixture 1 but in the form of crystalline amino acids. L-glutamic acid was added into each of the pre-mixtures so as to increase the total nitrogen to 2.816 g i.e. 17.6 g of protein equivalent which was comparable to that used by Dean and Scott (1965). Metabolizable energy of each ration was calculated by using the values of 4.48, 4.08 and 8.83 kcal M.E./g for casein or amino acids, corn starch and fat, respectively and has been recorded in Table 11.

For studying the effect of feeding TCA cycle intermediates on the status of biotin in the metabolism of chicks, it was considered desirable to deplete the chicks of biotin carried over in the embryos to a uniform level. Consequently all the chicks used for experiments reported in this section were fed a biotin-deficient basal ration for one week and then fed the experimental rations.

In trial 1, week-old chicks were divided into 3 groups of 33, 12 and 12 chicks and were fed rations 1, 2 and 3 respectively for the next 7 days. At 14 days of age, the chicks that had been fed ration 1, were divided into comparable groups of 6 chicks each and fed rations 1, 1a, 1b, 1c, and 1d for the next 7 days (Trial 2).

Trial 3 was conducted to study the possibility of replacing L-glutamic acid with some other sources of non-protein nitrogen. A basal ration (Ration 2a) was formulated by modifying ration 2, so as to supply 21.1 kcal M.E./g of protein. Using this basal a series of experimental rations were formulated in which all of the L-glutamic acid added to the basal ration was replaced isonitrogenously with

different sources of non-essential nitrogen, as indicated in Table 14. Differences in weights of rations resulting from the substitutions were adjusted with corn starch. The purified ration (Table 5) modified to contain vitamins at the same level as the basal ration was included as a positive control.

One hundred day-old male chicks were fed the biotin-deficient basal ration (Table 5) for one week. At 1 week of age the most uniform of the surviving chicks were divided into 7 comparable groups of 12 chicks each and fed the experimental rations for 14 days. Rates of growth were recorded at weekly intervals.

RESULTS

Trial 1 - The growth data obtained (Table 12) indicated that 10% of each of the EAA could be replaced with L-glutamic acid without any adverse effect on the growth of chicks. In fact the chicks fed the ration containing 90% EAA (Ration 2) showed a slight improvement in growth over chicks fed 100% EAA from the same sources (Ration 1).

Table 12. Effect of level and source of EAA on growth of chicks

Ration Number	Major Source of Amino Acids	Level of EAA (%)	Growth Rate During 2nd wk of age (g/wk)
1	casein	100	71
2	casein	90	79
3	crystalline amino acids	100	55

On the other hand the use of crystalline amino acids as the entire source of protein (Ration 3) reduced the rate of growth very markedly. The rates of growth were 10.1, 11.3 and 7.9 g/day for chicks fed

rations 1-3, respectively during second week of growth.

Trial 2 - The results of the second trial (Table 13) showed that the level of energy in the ration could be increased to give a ratio of 20.2 kcal of M.E./g of protein without affecting rate of growth (Ration 1a), however, when rations with wider calorie:protein ratios were used (Rations 1b, 1c and 1d) the rate of growth was decreased by 6, 11 and 11% respectively. Thus it was inferred that about 21 kcal M.E./g of protein would probably supply a slight excess of energy and would be high enough to minimize the degradation of amino acids.

Table 13. Effect of level of energy on the growth of chicks

Ration Number	Energy Level (kcal M.E./g of protein)	Growth Rate During 3rd wk of age (g/wk)
1	17.9	101
1a	20.2	101
1b	22.5	95
1c	24.1	90
1d	25.5	90

Trial 3 - The results obtained (Table 14) indicated that various sources of non-essential nitrogen used with the exception of DL-alanine were satisfactory substitutes for L-glutamic acid. In fact the use of ammonium bicarbonate, urea, ammonium acetate, 1/2 DL-alanine and 1/2 ammonium bicarbonate or L-glutamic acid in low protein rations gave growth rates of chicks essentially similar to

Table 14. Effect of source of non-essential nitrogen on growth of chicks

Ration Number ¹	Source of Non-essential Nitrogen	Level in the Ration ²	Avg Weekly Growth	
			2nd Week	3rd Week
1	L-glutamic acid	3.41	56	85
2	DL-alanine	2.06	44	76
3	1/2 DL-Alanine + 1/2 Amm. bicarbonate	+ 1.03 0.88	59	89
4	Ammonium bicarbonate	1.76	57	84
5	Ammonium acetate	1.78	60	95
6	Urea	0.67	58	80
7	High Protein Purified Ration	-	55	86

¹ Rations 1-6 were low protein rations and ration 7 was the purified ration (Table 5).

² In rations 1-6 the source of non-essential nitrogen supplied 0.324% N₂ i.e. about 11% of the total nitrogen in the ration.

that of chicks fed the purified ration (Table 5). When DL-alanine was used to replace the entire amount of added L-glutamic acid, the growth of chicks was depressed by 15%. For subsequent work it was decided to use ammonium bicarbonate as the source of non-essential nitrogen and to adopt ration 4 (Table 11) as the basal ration.

DISCUSSION

The results of this experiment indicated that, at the energy level used, the supply of each EAA in chick rations may be limited to 90% of the level recommended by Dean and Scott (1965) without any adverse effect. It was also observed that when a mixture of 14%

protein in casein and crystalline amino acids formed this EAA ratio, the balance of non-essential nitrogen requirement could be supplied by nitrogenous compounds such as ammonium bicarbonate, ammonium acetate or urea.

The study suggested that chicks have a limited capacity to utilize D-alanine. When 2.06% DL-alanine replaced 3.41% L-glutamic acid in the ration growth rate was depressed by 15% but when 1.03% DL-alanine and 0.88% ammonium bicarbonate were used growth rate was similar to that obtained when L-glutamic acid was used. A similar depression in growth rate of chicks, when D-alanine or DL-alanine was fed, has been reported by other workers. Adkins, Sunde and Harper (1962) found that addition of 1.25% D-alanine depressed the growth rate by 50%. Sugahara et al. (1967) reported that D-alanine at 1% level caused a slight growth retardation. More recently Renner (1969) reported that addition of 2.42 and 4.83% DL-alanine to diets depressed the growth of chicks by 33-58% and 92% respectively.

SUMMARY

1. A moderately high energy, low protein diet with optimum EAA balance, giving growth performance comparable to a high protein ration was developed for use in studies of the effect of TCA cycle intermediates on the status of biotin in the metabolism of chicks.

2. Rate of growth was not affected when 3.41% of L-glutamic acid in the ration was replaced isonitrogenously by ammonium bicarbonate, ammonium acetate or urea.

3. It appeared that the chick has a limited capacity to utilize D-alanine. Inclusion of DL-alanine in the ration at a level of 2.06% resulted in a 15% decrease in rate of growth but 1.03% DL-alanine along with 0.88% ammonium bicarbonate had no effect on rate of growth.

Experiment 2. Effect of feeding sodium succinate and citric acid on the status of biotin in the metabolism of chicks

OBJECT

The object of this experiment was to study the effect of feeding TCA cycle intermediates on the status of biotin in the metabolism of growing chicks.

EXPERIMENTAL

The basal ration used (Ration 4 - Table 11) contained ammonium bicarbonate as a source of non-essential nitrogen. This ration was a modification of Ration 4 (Table 14) in which the level of sodium chloride was increased from 0.6 to 0.88% to produce the same level of sodium as in 4% of sodium succinate. The experimental rations included combinations of four levels of biotin (0, 80, 320 and 640 $\mu\text{g}/\text{kg}$) and two levels of succinate-citrate supplement (0 and 10% of 2 sodium succinate:3 citric acid mixture). When succinate-citrate supplement was included in the ration it replaced an equal weight of corn starch.

One hundred and twenty, day-old male chicks were fed a biotin-deficient purified ration (Table 5) for one week, following which, the most uniform of the surviving chicks were divided into 8 groups of 12 chicks each and fed the experimental rations. At three weeks of age the chicks were weighed and examined for symptoms of biotin deficiency. Three chicks were selected at random from each of the groups and their livers were removed. Each liver was lyophilized separately and the resulting powder was assayed for PC activity.

RESULTS

The results obtained (Table 15) showed that inclusion of 4% sodium succinate plus 6% citric acid in rations fed to growing chicks

Table 15. Effect of feeding sodium succinate and citric acid on growth and liver characteristics of chicks

	Group Number							
	1	2	3	4	5	6	7	8
Nutritional treatments :-								
(a) Sodium ¹ succinate (%)								
(b) Citric acid (%)	-	-	-	-	4	4	4	4
(c) d-biotin ($\mu\text{g}/\text{kg}$)	0	80	320	640	0	80	320	640
Incidence of dermatitis (%)								
Mortality (%)	100	0	0	0	100	90	0	0
Avg wt gain, 7-21 days (g)	42	25	0	0	58	17	17	17
Avg liver wt (g)	44	107	145	143	69	66	107	116
Liver Protein (%)								
Avg PC Activity of liver ² (units/g)	21.2	21.9	24.4	23.6	21.8	21.7	22.9	23.2
	0.31	3.57	10.09	14.61	0.63	0.46	9.40	22.21
	±0.13	±1.42	±0.93	±2.81	±0.16	±0.06	±0.82	±1.44

1 Amounts of Na^+ , K^+ and Cl^- were the same in each ration and were adjusted by adding NaCl , KCl and KHCO_3 .

2 Values are means $\pm \text{SEM}$ of 3 samples in duplicate.

resulted in an increased incidence of dermatitis, an increased mortality rate and generally in a decreased rate of growth. Succinate-citrate supplementation appeared to improve rate of growth only in the absence of added biotin (Group 5 vs. Group 1) but rates of growth in both these groups were very low. All of the chicks fed biotin-deficient rations (Group 1 and 5) showed severe dermatitis. The addition of 80 μg of biotin/kg of ration (Group 2) completely alleviated the dermatitis but when succinate-citrate supplement was included along with this level of biotin, dermatitis occurred in 90% of the chicks. Thus it seems that 4% sodium succinate and 6% citric acid was not well tolerated by chicks and may have caused some metabolic disturbances.

Protein content of chicks liver was in the range of 21.2 to 24.4% but was not affected by either level of biotin or by the succinate-citrate supplement.

In general the PC activity of chick liver was proportional to the biotin level of the ration fed. The use of 640 μg of biotin/kg of ration increased the PC activity of chick liver very markedly above that in livers of chicks fed 320 μg of biotin/kg of ration, although the rates of growth in both cases were similar. Succinate-citrate supplementation seemed to decrease the PC activity in livers of chicks when biotin content of the ration was very low (80 $\mu\text{g}/\text{kg}$) but to increase the PC activity in chick livers when a high level of biotin (640 $\mu\text{g}/\text{kg}$) was available. These observations also, indicated that succinate-citrate supplementation caused some metabolic stress in growing chicks.

DISCUSSION

The inclusion of 4% sodium succinate and 6% citric acid in rations containing biotin apparently resulted in lower growth rate,

higher incidence of dermatitis and higher mortality rate as compared to groups fed equivalent rations without succinate-citrate supplement. Dakshinamurti and Mistry (1963) reported the feeding of rations containing 10% sodium succinate to normal and biotin-deficient chicks and rats but did not report any growth or mortality data. In a preliminary trial conducted prior to this study it was found that inclusion of 10% sodium succinate in purified rations with or without adequate biotin resulted in a very high mortality in day-old as well as three weeks old chicks.

The indication that growth rate of biotin-deficient chicks increased in response to succinate-citrate supplement suggested that, in biotin deficiency, the availability of C₄-dicarboxy acids might be the factor limiting performance of the chicks. A similar conclusion was drawn by Dakshinamurti and Mistry (1963), who reported that in chicks and rats the incorporation of labelled amino acids into tissue proteins in vivo was reduced in biotin deficiency but it was restored to normal levels when 10% sodium succinate was fed.

The increased PC activity in liver of chicks in response to increased biotin in the ration above 320 µg/kg is of interest. In view of the fact that PC activity may play an important role in a protein sparing action of carbohydrates (Varma and Mistry, 1967) and in lipogenesis (Ballard and Hanson, 1967), it may be understandable that PC responded to levels of dietary biotin higher than those resulting in a growth response. It may therefore be desirable to add biotin to poultry rations at levels higher than those required for maximum growth.

SUMMARY

The effects of feeding 4% sodium succinate and 6% citric acid in combination with four levels of biotin (0, 80, 320 and 640 $\mu\text{g}/\text{kg}$ of ration) in low protein rations for chicks were studied. The following results were obtained:-

1. The addition of succinate-citrate supplement to the rations fed resulted in an increased incidence of dermatitis, a decreased rate of growth and increased mortality except in the group fed rations without added biotin, in which case an increased rate of growth was noted.

2. The effects of succinate-citrate supplement on PC activity of chick liver were variable depending upon the level of biotin in the ration suggesting that high level of succinate-citrate used may have caused some metabolic disturbances.

3. In general PC activity in livers of chicks was proportional to biotin content of the ration fed. There was an indication that biotin requirement for maintaining maximum level of PC activity may be much higher than that required for optimal growth.

Experiment 3. Effects of feeding sodium succinate under conditions of limited biotin supply on the status of biotin in the metabolism of chicks

OBJECT

The purpose of this experiment was to study the effects of feeding graded levels of sodium succinate in combination with sub-optimal levels of biotin on growth, symptoms of biotin deficiency, liver protein content, PC activity and in vivo incorporation of ³²P into nucleic acids.

EXPERIMENTAL

For this study ration 4 (Table 11) was adopted as the basal. Combinations of three levels of sodium succinate (0, 2 or 4%) and two sub-optimal levels of biotin (80 or 120 µg/kg of ration) were compared with a biotin-deficient ration and a ration containing adequate biotin (640 µg/kg). Experimental rations were prepared by substituting required amounts of sodium succinate for an equal weight of starch and by adding the required amounts of d-biotin.

For one week, two hundred and twenty chicks were fed the biotin deficient purified ration (Table 5). At one week of age the most uniform of the surviving chicks were divided into 8 comparable groups of 24 chicks and fed the experimental rations. Group I was fed ad libitum but the other groups were pair-fed to the same level as Group 8 which showed minimal consumption and was fed ad libitum.

At 24 days of age, two groups of 4 chicks each were selected at random from each of the groups 2-8. These chicks were fed the respective rations ad libitum for one day. On the 25th day of age the chicks were killed and their livers were removed. Livers from

each group of 4 chicks were then homogenized together and lyophilized. The resulting powders were assayed in duplicate for PC activity.

At 29 days of age, the remaining chicks of the groups were fed ad libitum. The following day chicks were taken off feed for two hours and then eight chicks from each group were injected, intracardially, with saline solution containing 250 mc of sodium dihydrogen phosphate-³²P per ml, at the rate of 50 μ l per 25 g body weight. For the next four hours chicks were kept in groups of four in chick boxes and given feed and water ad libitum.

Four hours after injection, the chicks were killed by decapitation and their livers were removed. Each liver was weighed, put in a polyethylene bag and placed in boiling ethanol for 5 minutes to inactivate the nucleases (unpublished work cited by Munro and Fleck, 1966). The livers were then frozen in a methanol-dry ice bath and stored at -20 C.

Four livers from the same group were pooled to make a sample for isolation of nucleic acids. Two such samples from each treatment were examined for ³²P incorporation into nucleic acids. The RNA and DNA were isolated from the liver samples by the modified procedure of Fleck and Munro (1962) and levels of ³²P were determined using a liquid scintillation counter. Details of the procedure are presented in Appendix V.

Acid soluble phosphate was extracted from the liver samples by the method of Schmidt and Thanhauer (1945) and the phosphate content of the extracts was estimated by the method of Hurst (1964).

Relative incorporation of ³²P into nucleic acids was calculated as follows:-

$$\text{Relative Incorporation} = \frac{\text{dpm in RNA or DNA/g of liver}}{\text{dpm}/\mu\text{g of acid soluble phosphate in liver}}$$

The results obtained are presented in Table 16.

RESULTS

As the groups 2-8 were pair-fed growth rates were similar in all of the groups; weight gains during 7-28 days period ranged from 247 to 258 g. The growth rate of biotin-deficient chicks (Group 1) was much lower i.e. 141 g for the same period.

All of the chicks in the group fed the biotin-deficient ration developed severe dermatitis and mortality was very high (Group 1). Chicks in all other groups were free of symptoms of a biotin-deficiency and mortality rate was low.

Protein content of the liver ranged from 22.3-25.0% but did not appear to be influenced by levels of biotin or sodium succinate fed.

Under sub-optimal supply of biotin, PC activity in chick liver was directly proportional to the biotin level of the ration, i.e. when rations contained 80 or 120 μg of biotin/kg, the PC activities in livers were 11.09 units and 16.72 units/g of liver respectively. Increasing the biotin content of the ration of 640 $\mu\text{g}/\text{kg}$ i.e. well above the requirement for optimal growth, increased the PC activity in liver to 22.51 units/g of liver. Supplementation of rations containing 80 or 120 μg of biotin/kg, with 2 or 4% sodium succinate had no consistent effect on PC activity of chick liver.

Levels of biotin in the rations affected the incorporation of ^{32}P into RNA and DNA in chick liver. Relative incorporation of ^{32}P into RNA was 89.7, 149.6, 122.6 and 179.1 and into DNA was 12.0, 22.9, 26.9 and 29.0, respectively, when the rations contained 0, 80, 120 or

Table 16. Effect of feeding sodium succinate under limiting biotin supply on growth performance and PC activity and ^{32}P incorporation into nucleic acids in chick liver

Group Number	Nutritional Treatment		Properties of liver of 25 days old chicks			Incorporation of ^{32}P into nucleic acids in liver ¹		
	Sodium Biotin	Succinate	Mortality 7-28 days	Avg Wt	Protein	PC Activity ¹	RNA	DNA
1	0	0	50	141	--	--	89.7 \pm 4.2	12.9 \pm 0.2
2	80	0	0	248	8.8	22.3	11.10 \pm 0.43	149.6 \pm 24.2
3	80	2	4	253	9.6	24.0	7.90 \pm 0.01	22.9 \pm 0.8
4	80	4	4	258	9.7	25.0	10.84 \pm 1.01	138.8 \pm 16.7
5	120	0	0	247	8.9	24.6	16.72 \pm 0.54	19.5 \pm 1.2
6	120	2	0	247	10.1	23.5	13.18 \pm 0.06	24.8 \pm 7.3
7	120	4	0	255	10.0	24.6	16.89 \pm 1.04	22.6 \pm 3.1
8	640	0	0	248	8.1	23.3	22.51 \pm 1.52	24.8 \pm 7.3

¹ Values are average of two preparations \pm deviation from the average.

² Insufficient number of chicks in Group 1 for PC activity determinations because of high mortality.

640 μg of biotin/kg. The addition of 2 or 4% sodium succinate to rations containing 80 and 120 μg of biotin/kg apparently had no effect on the incorporation of ^{32}P into nucleic acids in the livers of chicks.

DISCUSSION

The results of this study support the observations mentioned earlier that when sub-optimal levels of biotin were fed PC activity in chick liver was directly proportional to the biotin content of the ration.

The inclusion of 2 or 4% sodium succinate in rations containing sub-optimal levels of biotin was very well tolerated but no consistent effects on PC activity of chick liver were noted. This suggested either that PC activation was not influenced by the dietary supply of C_4 -dicarboxy acids, when the biotin supply was limited or that dietary succinate failed to reach, in proper C_4 -dicarboxy form, the cellular site where C_4 -dicarboxy acids might be needed to exert an influence on PC activation.

A deficiency of biotin in the chick resulted in a marked reduction in the incorporation of ^{32}P into RNA and DNA of livers. No similar work has been reported with chicks but there are a few reports of related work with rats. Ricceri, Giuffrida and Cantone (1961) reported that in biotin-deficient rats, the incorporation of ammonium chloride- ^{15}N in vivo into pyrimidine and purine bases was decreased. Varma and Mistry (1966) observed that the biotin deficiency in rats reduced the incorporation of labelled glycine into adenine and guanine of visceral nucleic acids (RNA + DNA) but the incorporation into adenine and guanine of liver RNA was either unaffected or increased as compared to pair-fed normal rats. Caldara, Puddu and Marchetti (1967) noted

that the content of adenine, cytidine and guanine, free nucleotides in the liver of biotin-deficient rats, decreased very significantly as compared to rats fed biotin but RNA and DNA content and turn-over were not influenced by a biotin deficiency. Some of this work supports the view that biotin is necessary for nucleic acid synthesis while other work indicates that biotin-deficiency did not affect the nucleic acid metabolism in rats. Probable reasons for the seemingly contradictory results may be related to species variation and to differences in the metabolic state of the animals used. It is usually considered that synthesis of biotin by intestinal microflora is much greater in rats than in chicks and that the rat is less likely to become biotin-deficient. It is therefore possible that the biotin deficient rats used by Varma and Mistry (1966) and Calderara et al. (1967) were not as severely deficient in biotin as the chicks used in this experiment. Also Varma and Mistry (1966) restricted feed intake of control rats to the level consumed by their biotin-deficient counterparts. This practice would have caused the normal rats to suffer oscillations of starvation and consequently such animals would not be in normal physiological condition and might have developed some chronic adaptation. Very recently, Patel and Mistry (1969) have reported that metabolic alterations were induced in animals as a result of food restrictions. Some of the variability reported may have arisen because the methods employed measured somewhat different metabolic functions. The studies in which labelled ammonium chloride (Ricceri et al., 1961) or labelled glycine (Varma and Mistry, 1966) were used would determine de novo synthesis of nucleotides and their incorporation into nucleic acids, whereas studies involving ^{32}P incorporation

into nucleic acids would measure the total effect of de novo synthesis and turnover of nucleotides. Thus ^{32}P incorporation into nucleic acids should give an indication of the physiological activity of the tissue. In the present study, the relative incorporation of ^{32}P into RNA and DNA in livers of biotin-deficient chicks was only 50% and 44% of the normal chicks, respectively. In the chicks fed sub-optimal levels of biotin, the incorporation of ^{32}P into nucleic acids was intermediate to biotin-deficient and chicks fed adequate biotin. Thus it is apparent that biotin plays a role in the metabolism of nucleic acids in chick liver.

The results of this study also suggested that there was no direct relationship between PC activity of chick livers and their capacity to incorporate ^{32}P into nucleic acids. Also, the inclusion of sodium succinate in rations containing sub-optimal levels of biotin failed to enhance ^{32}P incorporation into nucleic acids of liver. Further studies along similar lines would be required to establish the role of biotin in nucleic acid metabolism. A study of carbamyl phosphate synthetase in relation to the effect of biotin nutrition on nucleic acid metabolism in chick liver might be desirable because it is involved in the synthesis of nucleic acids and there have been reports that it may be a biotin dependent enzyme (Review of Literature - Section C).

SUMMARY

Effects of feeding 0, 2 or 4% sodium succinate in combination with sub-optimal levels of biotin on deficiency symptoms, chick liver protein, PC activity and incorporation of ^{32}P into RNA and DNA were compared with results of chicks fed rations deficient or adequate in biotin. The following results were obtained:-

1. All of the chicks fed biotin deficient rations developed typical deficiency symptoms but chicks in other groups were free of any visible deficiency symptoms. No adverse effects from the addition of 2 or 4% sodium succinate to the ration were noted.

2. In chicks fed rations without added succinate, the PC activity of liver increased as the biotin supply was increased. Feeding of sodium succinate apparently had no effect on PC activity of chick liver.

3. In the chicks deficient in biotin, the relative incorporation of ^{32}P into RNA and DNA in livers was only 50% and 44% of the normal chicks fed biotin, respectively. In chicks fed sub-optimal levels of biotin, the relative incorporations of ^{32}P were intermediate to those of biotin-deficient and chicks fed adequate biotin. However, the feeding of sodium succinate apparently had no effect on ^{32}P incorporation into nucleic acids in chick liver.

GENERAL DISCUSSION

The methods of Utter and Keech (1963) for estimation of PC activity in crude preparations of liver was improved by including a GOT trap in the assay mixture to remove oxaloacetate from the site of the reaction and by establishing conditions under which PC was relatively more stable. Using the improved assay procedure, a linear increase in PC activity of chick liver, in response to increased biotin supply over the range of 80-320 $\mu\text{g}/\text{kg}$ of ration, was established. Thus it was inferred that PC activity of liver could be employed as a parameter for assessing the biotin status of poultry and for estimating the net effect of the ration on the supply of metabolically active biotin.

The performance of turkey poult's was affected by the source of protein supplement used. When meat meal and fish meal formed the entire protein supplement in turkey starter ration, poult's developed typical symptoms, characteristic of the syndrome reported by other workers (Review of Literature, Section B) and gave much lower rate of growth than poult's fed about one half or almost all of the protein supplement as soybean meal. The better performance of poult's fed rations containing soybean meal may be due to higher biotin content and a better amino acid make up of the ration. Supplementation of the ration containing only fish-meat meal as the supplemental protein, with biotin alleviated all of the deficiency symptoms and resulted in doubling the PC activity of poult liver but failed to improve the rate of growth. These observations suggested that either the meat-fish meal supplement was also lacking in some growth factor other than biotin or its use resulted in an imbalance of certain nutrients and thereby suppressed the growth of poult's.

It is of interest that in both chicks and poult the PC activity in livers increased in response to levels of dietary biotin higher than those resulting in a growth response. In view of the suggestions that PC activity may play an important role in the protein sparing action of carbohydrates (Varma and Mistry, 1967) and in lipogenesis (Ballard and Hanson, 1967), the above observation is of great significance and it may be desirable to add biotin, to poultry rations at levels higher than those required for maximum growth.

The sequence of appearance of symptoms in biotin-deficient chicks and effects of graded levels of biotin on PC activity of chick liver suggested that the usage of biotin in chicks for alternate functions may have priorities depending upon the extent of its availability. For example when biotin supply is very limited, most of it may be used for the synthesis of pyruvate holocarboxylase needed to sustain life; when a little more biotin is available, other biotin dependent enzymes may be enhanced to alleviate the metabolic disorders which result in visible deficiency symptoms and when still higher levels of biotin are available it may then be used to activate more PC activity to supply essential metabolites required for growth and normal metabolic activity of the body. This conclusion seems to be substantiated by the observation that the addition of sub-optimal levels of biotin to chick rations resulted in a smaller increase in PC activity in livers of chicks fed high protein ration (Expt. 2, Section I - Part B) than when low protein rations were fed (Expt. 2 and 3, Section III). It may be theorized that when high protein rations were fed a larger proportion of biotin would be used for activating other biotin-dependent enzymes needed to catabolize the excess of amino acids than when lower

protein rations were fed and, as a consequence, less biotin would be available for activating PC activity. The possibilities of changes in the biosynthesis of biotin by intestinal microflora and variations in the supply of residual biotin from different lots of vitamin-test casein can not be eliminated. In fact these variations might be the reason for variations in PC activity in livers of chicks fed the same level of biotin (80 $\mu\text{g}/\text{kg}$ of ration) in Expt. 2 and 3, Section III.

A deficiency of biotin resulted in an impairment of nucleic acid metabolism in chick liver. In biotin-deficient chicks the relative incorporation of ^{32}P into RNA and DNA of liver was reduced to 50% and 44% respectively of that in chicks fed adequate biotin. Even in chicks fed sub-optimal levels of biotin the relative incorporation of ^{32}P into liver nucleic acids was slightly less than in those receiving adequate biotin. However, there was no direct relationship between PC activity of chick livers and their capacity to incorporate ^{32}P into nucleic acids.

As the basic function of PC is to replenish the TCA cycle intermediates withdrawn for the biosynthesis of a variety of metabolic products, the effect of feeding TCA cycle intermediates to chick was studied. Addition of 4% sodium succinate and 6% citric acid to rations for growing chicks resulted in some metabolic disturbances but 4% sodium succinate alone was very well tolerated. The inclusion of 2 or 4% sodium succinate in the rations containing sub-optimal levels of biotin (80 and 120 $\mu\text{g}/\text{kg}$) apparently had no effect on PC activity and in vivo ^{32}P incorporation into RNA and DNA of chick liver.

Although inclusion of succinate did not affect nucleic acid metabolism in the present study, yet there might be an effect upon

addition of succinate to rations devoid of biotin. In addition, a study of the effect of feeding graded levels of biotin on carbamyl phosphate synthetase and its relationship to nucleic acid metabolism would be of interest, because it is involved in the de novo synthesis of nucleotides and there have been reports that carbamyl phosphate synthetase may be a biotin-dependent enzyme.

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APPENDIX I

Procedure for estimating protein by biuret reaction

Liver homogenate or suspension of lyophilized powder was diluted with 2.5 N NaOH to a final concentration of 1N NaOH, so that the final solution contained 10-20 mg of protein per ml. This suspension was kept at room temperature for 30 min, with occasional shaking without permitting any foam formation. Duplicate samples of 0.1 ml or 0.2 ml of the solution were taken in stoppered centrifuge tubes and diluted to 3.0 ml. To each tube, 3.0 ml of biuret reagent was added and contents mixed immediately by inversion.

The reaction was allowed to proceed for 25 min at room temperature at which time 2.0 ml of diethyl ether was added to each tube and the tube was shaken vigorously for 15-20 sec. The tubes were then centrifuged at 450 x g for 5 min and the ether layer was discarded.

Forty five min after adding biuret reagent, the intensity of colour in the aqueous layer was measured at 540 m μ , in a spectronic 20 or a Beckman spectrophotometer DB-G model.

A standard curve was prepared by the same procedure using 0.2 to 4.0 mg of bovine albumin.

Modified biuret reagent

One and a half gram of cupric sulphate ($CuSO_4 \cdot 5H_2O$) and 6 g of sodium potassium tartrate were dissolved in 500 ml of water, to this solution 275 ml of 10% NaOH solution was added with swirling and volume made up to 1 litre.

APPENDIX II

Procedure for estimating pyruvate carboxylase (PC)(a) Extraction of PC activity from liver powder

Lyophilized liver powder was homogenized with 10 volumes (ml/g) 50 mM tris-HCl buffer pH 7.6, at room temperature, using an electrically operated Teflon homogenizer. After 10 min, the suspension was centrifuged at 1000 x g for 2-3 min. The supernatant was assayed for PC activity within the next 30 min. Just before assay, the supernatant was diluted with a solution containing 100 mg albumin per ml, pH 7.6. The amount of albumin solution used varied depending upon the PC activity of the preparation.

(b) Assay of PC activity

The assay mixture contained 100 mM tris-HCl buffer, pH 7.8; 20 mM NaHCO₃ (about 16 to 40 dpm per μ mole); 10 mM MgCl₂; 10 mM potassium pyruvate; 1 mM acetyl CoA; 5 mM ATP; 1.2 mM Cleland's reagent; and 10 mM glutamate. For each assay 0.5 ml of assay mixture was used and it contained 2 units of GOT and 10-50 μ l of lyophilized liver extract. For each preparation, one control tube without pyruvate was included.

To start the reaction enzyme was added to successive tubes at 30 sec interval, the tubes were stoppered and placed in a water bath at 30 C. Exactly after 5 min the reaction was stopped by adding 0.05 ml of 5 N HCl. The tubes were then placed in a hood with a strong exhaust system. Two drops of amyl alcohol were added to each tube, to check frothing, and CO₂ was bubbled through the mixture at a slow rate for 30 min to remove CO₂-¹⁴C. The contents of each tube were then neutralized by adding 0.05 ml of 5N NaOH to each tube.

(c) ^{14}C counting by gel-suspension technique

Vials were filled with Carb-o-sil, about 12 ml of scintillation fluid was added to each vial and after shaking thoroughly, the vials were counted for background. Vials giving the same background counts and similar efficiency were used for control and test of a certain determination.

The neutralized assay mix was quantitatively transferred into these vials, the tube was washed thoroughly with scintillation fluid and the washings also transferred to the vial. The vials were shaken vigorously to suspend the aqueous phase evenly and then counted in Nuclear Chicago Analyzer I, using the following setting:-

	Channel	
	A	C
Attenuation	C_{550}	D_{550}
Lower Discriminator	0.5 volts	0.5 volts
Upper Discriminator	2.9 volts	9.9 volts
Integrator	L - U	L - U

With unquenched standards, these settings gave an efficiency of 87.7%; samples were usually counted with efficiency of 70 - 75%.

Scintillation Fluid

Dioxane	600 ml.
Anisole	100 ml.
Dimethoxyethane	100 ml.
PPO (2, 5-diphenyloxazole)	4.8 gm.
POPOP (1, 4-bis [2-(5-phenyloxazolyl)] benzene)	240 mg.

(d) Estimation of enzyme activity

For each preparation, one control (without potassium pyruvate) was included and dpm of control was subtracted from that of the test run, to assess the dpm fixed by the enzyme into organic form.

The specific activity of the sodium bicarbonate-¹⁴C used was estimated by counting 0.2 ml duplicate samples of the premix (containing all the constituents of the assay mixture, other than potassium pyruvate and enzyme), taken just before and immediately after completing each set of assays.

The activity of pyruvate carboxylase was calculated as follows:-

unit = μ moles of bicarbonate-¹⁴C incorporated into non-volatile form, in the presence of pyruvate at 30 C.

$$\text{units/g of protein} = \frac{\text{dpm fixed} \times \text{mg of protein/g of liver powder}}{5 \times \text{dpm}/\mu\text{moles of NaH}^{14}\text{CO}_3 \times \text{mg of liver powder/assay}}$$

$$\text{units/g of liver} = \frac{\text{units/g of protein} \times \% \text{ protein in liver}}{100}$$

APPENDIX III

Procedure for preparing acetyl-CoA

The entire procedure was carried at 2-5 C and is described for a lot of 1 ml of 10 mM acetyl-CoA solution.

Ten mg of coenzyme A was dissolved in 0.6 ml of water and 0.1 ml of 1 M KHCO₃ was added to it. The solution was tested for the presence of free sulfhydryl groups which gave pink colour on the nitroprusside paper.

One drop of acetic anhydride was added and the pH maintained at 7.5 for about 15 min until the free sulfhydryl groups had disappeared. The solution was then acidified with 1N HCl to a pH of 3 and extracted 4 times with 3 volumes of cold diethyl ether, to remove free acetate and unused acetic anhydride.

The aqueous layer was again tested for free sulfhydryl groups which should be absent at pH 7.3 i.e. 1 M KHCO₃ but should appear at pH 10 i.e. 1N NaOH. The solution was then adjusted to pH 5 with very dilute NaOH and N₂ gas bubbled through it, to remove any remaining ether. The solution was made to a volume of 1.0 ml with distilled water and stored at -10 C.

Preparation of nitroprusside paper

One gram of sodium nitroprusside was dissolved in a minimum amount of water and the volume was made upto 75 ml with methanol. Small pieces of filter paper were dipped in this solution and dried in air.

APPENDIX IVComposition of amino acids mixtures

<u>Amino Acids*</u>	<u>Mix I</u> (g)	<u>Mix II</u> (g)
L - Arginine. HCl	67.34	74.80
L - Histidine. HCl.H ₂ O	--	23.05
L - Lysine. HCl	--	78.75
L - Tyrosine	--	35.40
DL - Tryptophane ¹	3.00	25.30
DL - Phenylalanine ²	--	38.25
DL - Methionine ²	8.80	30.95
L - Cystine	30.00	19.70
DL - Threonine ¹	2.80	73.15
L - Leucine	--	67.50
DL - Isolencine ¹	52.00	90.00
DL - Valine ³	--	61.50
Glycine	132.00	90.00
L - Proline	--	56.25
	295.94	764.55

* As suggested by Sugahara *et al.* (1967) the D - form of amino acids have been assigned the following nutritive values:-

1. Small nutritive value.
2. Same nutritive value of the L-form.
3. One half the nutritive value of the L-form.

APPENDIX VProcedure for the isolation of nucleic acids

Composite samples of liver were homogenized in 19 volumes (ml/g) of ice cold water in a Waring blender. A sample of 400 ml of the homogenate was placed in a plastic bottle, 40 ml of 10.5 N perchloric acid (PCA) was added by stirring, and the mixture was allowed to stand for 15 min at 2-5 C. The suspension was then centrifuged at 800 x g for 10 min, and the supernatant was discarded and the precipitate was washed, twice with 0.7N PCA.

Lipids were extracted from the precipitate by suspending it in 140 ml lots of the following solvents and centrifuging at 800 x g for 10 min:-

- (a) Cold acetone
- (b) Ethanol
- (c) Ethanol: chloroform (3:1)
- (d) Ethanol: ether (3:1)
- (e) Ether

Extraction of RNA

After the lipids were extracted, the precipitate was transferred into glass bottles, 200 ml of 0.3 N KOH at 37 C was added and the mixture was incubated at 37 C for one hour. The bottles were then chilled, their contents neutralized with 10N PCA and acidified by adding 1 volume of 10 N PCA per 19 volumes of mixture.

The suspension was then centrifuged at 800 x g for 10 min and the supernatant was separated. The precipitate was washed twice with 0.5 N PCA and the washings were combined with the supernatant and made up to 250 ml with 0.5 N PCA. This solution contained RNA.

Extraction of DNA

The RNA free precipitate was suspended in 60 ml of 1 N PCA and digested at 80 C for 30 min. The suspension was then centrifuged at 800 x g for 10 min and the supernatant was collected. The precipitate was again extracted with 40 ml of 1 N PCA, the second supernatant was added to the first and the volume was made up to 100 ml. This solution contained DNA.

Counting for ^{32}P

Aliquots from isolated RNA and DNA solutions were counted by the internal standard method of liquid scintillation counting; using 20-1 dynamic range of channel B, with the following setting:-

Window at G 620.

Lower attenuation at 0.5 volts.

Upper attenuation at 9.9 volts.

Integrator at L-U.

LIST OF ABBREVIATIONS¹ USED

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Avg	Average
c	Curie (3.7×10^{10} disintegrations/second)
C	Degree, Celsius
C ₄	Four carbon
-CoA or -SCoA	Coenzyme A
DNA	Deoxyribonucleic acids
dpm	Disintegrations per minute
EAA	Essential amino acid(s)
GOT	Glutamate oxaloacetate transaminase
G-PDH	Glyceraldehyde-3-phosphate dehydrogenase
g	Gram
x g	Gravity, centrifugal
kcal	Kilocalorie(s)
kg	Kilogram
M.E.	Metabolizable energy
μ	Micro
m	Milli
M	Molar
NAD(P) ⁺	Nicotinamide adenine dinucleotide (phosphate), oxidized form
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate), reduced form
PC	Pyruvate carboxylase
PEP	Phospho-enol pyruvate
Pi	Inorganic orthophosphate
PPi	Inorganic pyrophosphate
RNA	Ribonucleic acids
SEM	Standard error of means
TCA cycle	Tricarboxylic acid cycle
wk	Week(s)
wt	Weight

¹ As described in Style Manual for Biological Journals and Journal of Biological Chemistry.

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